ASSESSING BROOK STICKLEBACK (*CULAEA INCONSTANS*) AS A BIOINDICATOR FOR ENDOCRINE DISRUPTING COMPOUNDS IN AQUATIC ENVIRONMENTS

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ABSTRACT

Endocrine disrupting compounds (EDC) are environmental contaminants that disrupt reproduction, development and behaviour in aquatic organisms. A thorough evaluation of the impacts of EDCs on aquatic organisms is currently limited by a lack of robust biomarkers in small model fish, particularly for assessing EDCs with (anti-)androgenic activity. Male sticklebacks build nests using spiggin, an androgen-responsive glycoprotein, which can be used to assess (anti-)androgenic exposure. EDC assessment in the field using threespine stickleback and the spiggin biomarker is limited to coastal and estuarine environments. However, their freshwater relative, brook stickleback (*Culaea inconstans*), also possess spiggin and their widespread distribution suggests that they may have applications as a bioindicator of EDCs in freshwater systems. Therefore, the overall objective of this thesis was to determine if brook stickleback are a suitable bioindicator species for EDCs by evaluating their response and sensitivity to estrogenic and (anti-)androgenic chemicals.

Basal transcript levels of spiggin in kidney and vitellogenin in liver were first measured in wild-caught brook stickleback using qPCR and found to be differentially expressed in males and females. Brook stickleback were then exposed to two model compounds, 17α-ethinylestradiol (EE2) and 17α-methyltestosterone (MT), at 1, 10 and 100 ng/L for 21 days (sampled at 7 and 21 days) via static-renewal to determine the responsiveness of these transcripts to exogenous hormones. The effect of hormone exposure on condition factor, organosomatic indices and histopathology of kidneys was also measured. Exposure to MT and EE2 significantly induced spiggin and vitellogenin transcripts in female kidneys and male livers, respectively. Exposure to EE2 also significantly increased the hepatosomatic index in females after 7 days and in both sexes after 21 days whereas the gonadosomatic index was reduced in females after 21

days. An increase in kidney epithelium cell height was also observed in MT-exposed females and males after 7 days. These results mirror those of threespine stickleback and suggest that brook stickleback are responsive to androgenic and estrogenic chemical exposure and more specifically, possess quantifiable and sensitive biomarkers for exposure to compounds with androgenic activity.

In a third experiment, female fish were co-exposed to MT at 500 ng/L and an anti-androgen (flutamide; FL) at 25, 150 and 250 μg/L for 14 days (sampled at 4 and 14 days) to validate this bioassay for the evaluation of anti-androgens using the same endpoints as in the previous two experiments. In females, exposure to MT increased spiggin transcript levels and nephrosomatic index (NSI) but co-exposure to FL did not result in a significant suppression of these endpoints because of high inter-individual variability. In males, exposure to MT increased NSI and co-exposure to FL resulted in a reduction in this endpoint, illustrating anti-androgenic effects. Although the response of brook stickleback to hormone exposure was endpoint-specific and was at times lower than other small model fish species, the ability to simultaneously assess estrogenic and (anti-)androgenic chemical exposure in a single fish using quantitative endpoints is an advantage exclusively held by members of the stickleback family. The results of this thesis suggest that brook stickleback hold promise as an additional small fish model for the evaluation of EDCs, with potential application in EDC biomonitoring in the freshwaters of North America.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

(anti-)	refers to both an anti-androgen/androgen and anti-estrogen/estrogen
°C	degree Celsius
µg/L	microgram per litre
11-KT	11-ketotestosterone
AFSS	Androgenized Female Stickleback Screening Assay
ANOVA	analysis of variance
AR	androgen receptor
CF	condition factor
cDNA	complimentary deoxyribonucleic acid
EDC	endocrine disrupting compound
E2	17β-estradiol
EE2	17α-ethinylestradiol
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
ERE	estrogen response element
EROD	ethoxyresorufin-O-deethylase
FL	flutamide
GnRH	gonado-releasing hormone
GtH1	gonadotropin hormone I
GtH2	gonadotropin hormone II
GSI	gonadosomatic index
HPG	hypothalamus-pituitary-gonadal
HSI	hepatosomatic index
KEH	kidney epithelium cell height
LOEC	lowest observable effects concentration
MT	17α – methyltestosterone
mRNA	messenger ribonucleic acid
ng/L	nanograms per litre
NOEC	no observable effects concentration
NSI	nephrosomatic index
NSERC	National Science and Engineering Research Council
OECD	Organization for Economic Co-Operation and Development
PAH	polycyclic aromatic hydrocarbons
POP	persistent organic pollutants
PPCP	pharmaceuticals and personal care products
PPME	pulp and paper mill effluent
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RPL-8	ribosomal protein L8
US EPA	United States Environmental Protection Agency
WWTPE	wastewater treatment plant effluent

PREFACE

Chapter 1 of this thesis is a general introduction and Chapter 4 is a general discussion with overall conclusions. Chapter 2 and 3 are organized as manuscripts to be published in scientific journals. Therefore, there may be content that is repeated between the introduction and materials and methods sections across these chapters. Chapter 2 has been submitted to *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* whereas Chapter 3 is being prepared for submission to *Aquatic Toxicology*.

CHAPTER 1

1. GENERAL INTRODUCTION

1.1 Endocrine disruption in the aquatic environment

The potential for chemicals to disrupt the endocrine system in humans and wildlife has been a cause for concern for several decades (Colborn et al., 1993; Tyler et al., 1998). These chemicals are referred to as endocrine disrupting compounds (EDC) and, ultimately end up in the aquatic environment via effluent discharge or agricultural and storm water runoff (Bolong et al., 2009; Durhan et al., 2006; Sumpter, 2002). It is not unexpected then, that several of the documented cases of endocrine disruption are in aquatic organisms, particularly fish (Sumpter and Johnson, 2008). Therefore, regulatory agencies, including the United States Environmental Protection Agency (US EPA) and the Organization for Economic Co-Operation and Development (OECD), have established test protocols to assess EDC exposure and effects in fish (OECD, 2011, 2009; US EPA, 2009). Until recently, EDC assessment focussed on chemicals with estrogenic activity (Goksøyr, 2006; Larkin et al., 2003; Mills and Chichester, 2005; Rotchell and Ostrander, 2003). However, the discovery of masculinized female fish in the aquatic environment and detection of compounds with (anti-)androgenic activity in sewage effluent across the UK has more recently prompted the development of assays to assess (anti-)androgenic exposure in fish (Howell et al., 1980; Jobling et al., 2009).

1.2 Endocrine disrupting chemicals

EDCs are any chemical or mixture whose primary action alters the endocrine system resulting in an adverse effect on homeostasis, reproduction, development, and/or behaviour

(Campbell et al., 2006; Goksøyr, 2006). A large number of diverse compounds have been identified as having endocrine disrupting potential, including pharmaceuticals and personal care products (PPCPs), phytoestrogens, pesticides, persistent organic pollutants (POPs), polycyclic aromatic hydrocarbons (PAHs), and industrial chemicals such as bisphenol-A (Campbell et al., 2006; Daston et al., 2003; Mills and Chichester, 2005). These chemicals originate from several sources but end up in the aquatic environment as a result of surface runoff, atmospheric deposition, and effluent discharge into receiving waters (Campbell et al., 2006; Sumpter, 2002).

1.2.1 Aquatic sources of EDCs

Pulp and paper mill effluent (PPME), wastewater treatment plant effluent (WWTPE), and agricultural runoff are leading sources of EDC pollution in the aquatic environment (Durhan et al., 2006; Sumpter, 2002). PPME contains numerous chemicals, including chlorinated lignins, resin acids, POPs such as phenols, dioxins and furans, and also organic compounds such as phytoestrogens (Ali and Sreekrishnan, 2001). Exposure to PPME predominantly results in androgenic effects in fish (Sumpter, 2002; Wartman et al., 2009). Intensive livestock operations are also a source of androgenic chemicals. For example, trenbolone acetate is a growth promoter routinely used in this industry and its metabolites (17β- and 17α-trenbolone) are excreted by livestock and enter watersheds via runoff (Brooks et al., 2006; Durhan et al., 2006; Liu et al., 2011). In several laboratory exposures, the androgenic activity of 17β-trenbolone has resulted in masculinized female fish (Ankley et al., 2003; Battelle, 2003; Seki et al., 2006). Alternatively, WWTPE contains industrial and domestic chemicals, including PPCPs such as 17α-ethinylestradiol (EE2; an estrogenic contraceptive), various antibiotics, surfactants (Brooks et al., 2006) and is classified as causing primarily estrogenic effects in fish (Bolong et al., 2009; Matthiessen and Johnson, 2007). However, substantial anti-androgenic activity has recently been

detected in WWTPE from several facilities in England at levels associated with anti-androgenic effects in fish (Jobling et al., 2009; Johnson et al., 2007). Despite this, the impact of anti-androgenic exposure in fish is relatively unknown. Other sources of EDCs include septic tank soakaways, landfill leachate, roadway runoff, and pesticide application (Bolong et al., 2009; Campbell et al., 2006). Therefore, there are several sources for (anti-)androgenic pollutants in the aquatic environment and this warrants investigation into their potential to cause adverse effects in fish.

1.2.2 Mechanisms of EDC action

The hypothalamus-pituitary-gonadal (HPG) axis is a series of nervous and endocrine organs upon which EDCs can exert their effects (Ankley and Johnson, 2004). In fish, the HPG axis consists of several negative and positive feedback loops that up- and down-regulate development, growth and reproductive processes (Ankley and Johnson, 2004). Briefly, an external or internal cue stimulates the hypothalamus which releases gonadotropin-releasing hormone (GnRH). GnRH then prompts the release of gonadotropin hormone I and II (GtH1, GtH2) from the pituitary, which are similar to mammalian follicle stimulating hormone (FSH) and luteinizing hormone (LH) in humans, respectively (Arukwe, 2001). Gonadotropin hormones then trigger sex steroid production by ovaries or testes which result in gonad maturation and subsequent ovulation and sperm release in females and males, respectively (Arukwe, 2001). Sex steroids also stimulate other processes, including the production of sex-specific proteins involved in reproduction or oogenesis, development of secondary sexual characteristics (in some species), behavioural alterations, and courting behaviour (Arukwe, 2001).

EDCs can act on the HPG axis through a variety of mechanisms (Arukwe, 2001; Sumpter, 2002). They may mimic a hormone resulting in agonistic effects on cellular processes

(Arukwe, 2001; Goksøyr, 2006). Additionally, EDCs can bind to and block receptor binding sites thereby blocking endogenous hormones from binding and interfering with cellular pathways (Arukwe, 2001). This may cause an increase in endogenous hormone metabolism and decreased circulating hormone concentrations (Van Der Kraak et al., 1998). In both of these cases, the EDC shares a similar structure and shape to endogenous hormones (Arukwe, 2001). EDCs may also increase or decrease estrogen or androgen receptor (AR) expression or alter their function (Goksøyr, 2006). Alternatively, EDCs can act directly on hormones to interfere with hormone synthesis, metabolism, transport, secretion or degradation (Arukwe, 2001; Goksøyr, 2006; Rotchell and Ostrander, 2003). Lastly, EDCs may act indirectly to interfere with endocrine function by altering the expression of genes involved in reproduction (eg. vitellogenin) and thus DNA transcription and translation (Larkin et al., 2003; Sumpter, 2002). Emerging research indicates that the indirect action of chemicals on the endocrine system (eg. via receptor cross-talk; Diamanti-Kandarakis et al., 2009) or through non-receptor mediated effects on other cellular factors (such as protein kinases; Gore, 2007) may also contribute to endocrine disruption in fish.

1.2.3 Effects of (anti-)estrogenic EDCs in fish

In fish, EDCs can elicit effects across multiple levels of biological organization through their action on the HPG axis. In female fish, exposure to estrogenic chemicals may cause premature maturation or, in already mature fish, may lead to increased energy expenditure and lowered reproductive fitness (Arukwe, 2001). Sustained GtH1 synthesis, from persistently elevated plasma estrogen levels, may be the mechanism underlying premature maturation or prolonged reproduction in females (Arukwe, 2001). In male fish, exposure to estrogenic chemicals can lead to a suite of effects related to feminization, including delayed or absent

secondary sexual characteristics, altered reproductive behaviour, and delayed or absent testicular development (Arukwe, 2001; Giesy and Snyder, 1998). Overall, these effects can result in reduced reproductive fitness (Arukwe, 2001; Giesy and Snyder, 1998). A widespread response observed in several male fish species is the production of vitellogenin, a female egg yolk precursor (Angus et al., 2002; Ankley and Johnson, 2004; Arukwe, 2001; Goksøyr, 2006; Hutchinson et al., 2006). Male fish retain the cellular equipment necessary to produce vitellogenin, although endogenous estrogen levels are not high enough to stimulate production under basal conditions (Ankley and Johnson, 2004). Also, unlike females, males do not have an efficient method to eliminate vitellogenin from their body (Ankley and Johnson, 2004). Therefore, vitellogenin synthesis in males can cause liver hypertrophy and hyperplasia, kidney dysfunction, and organ failure as the protein accumulates in these organs (Ankley and Johnson, 2004; Arukwe, 2001; Hutchinson et al., 2006). The increased metabolic demand associated with vitellogenin production in female and male fish exposed to estrogenic EDCs also diminishes energy stores which can lead to lowered reproductive fitness (Arukwe, 2001). In females, exposure to anti-estrogenic chemicals can cause a decrease in the production and deposition of vitellogenin and zona radiata (egg shell) proteins resulting in eggshell abnormalities, reduced hatch rates, and reductions in fry survival (Ankley et al., 2009; Arukwe, 2001).

1.2.4 Effects of (anti-)androgenic EDCs in fish

Exposure to (anti-)androgenic chemicals also causes adverse effects in fish (Arukwe, 2001). The first prominent case of androgen disruption was reported in a wild mosquitofish (*Gambusia a. holbrooki*) population - females grew a modified anal fin called a gonopodium normally only observed in males and used for copulation (Ellis et al., 2003; Howell et al., 1980). Female mosquitofish also displayed reproductive behaviour typical of males including chasing

and gonopodia swinging and thrusting (Howell et al., 1980). Subsequent research determined that masculinized female mosquitofish were present at other sites located near PPME outfalls and, it was demonstrated that androgenic activity from PPME was to blame for the effect (Ellis et al., 2003; Parks et al., 2001). A suite of effects have now been reported in several species of small bodied fishes exposed to androgenic chemicals including masculinization, reduced or inhibited spawning, decreased fecundity, and decreased fertility (Ankley et al., 2001; Kang et al., 2008; OECD, 2009). However, endocrine disruption in wild fish has yet to be definitively linked to exposure to (anti-)androgenic chemicals; but, statistical modelling suggests that (anti-)androgenic activity in WWTPE may be at sufficient levels to adversely affect fish in effluent receiving waters (Jobling et al., 2009). Furthermore, Jobling et al. (2009) proposed that exposure to estrogenic- and anti-androgenic chemicals (rather than estrogenic chemicals alone) may be responsible for feminization of wild fish populations. For example, in laboratory studies exposure to estrogenic and anti-androgenic chemicals resulted in a similar suite of effects in small fish, including reduced secondary sexual characteristics, lowered sperm count, reduced fecundity, reduced fertility, altered body coloration, and altered courtship and nesting behaviour (Ankley and Johnson, 2004).

Potential long-term effects from EDC exposure include population collapse and the associated impacts at the community and ecosystem levels. An experiment by Kidd et al. (2007) was the first to explicitly link EDC exposure to population level effects. A population of fathead minnows (*Pimephales promelas*) collapsed following long-term exposure to EE2 in a whole-lake experiment (Kidd et al., 2007). Males from this lake displayed intersex whereas gonad development was delayed in females (Kidd et al., 2007). However, the population recovered in the years immediately after EE2 administration ended (Blanchfield et al., 2015). Nevertheless,

linking EDC exposure to population-level effects remains difficult because of the complexity of the endocrine system and the chemical mixtures thought to result in alterations to this system (Giesy and Snyder, 1998; Van Der Kraak et al., 1998). Research has linked estrogenic chemical exposure to adverse effects in wild-fish populations but the same link has yet to be made between (anti-)androgenic chemicals and population-level effects. An inability to distinguish (anti-)androgenic effects in wild fish may be related to a lack of sensitive and quantifiable (anti-)androgenic biomarkers. In order to fill this knowledge gap, developments need to continue to be made in identifying and calibrating a standardized test using small fish models that can provide more information on (anti-)androgenic effects in wild fish populations.

1.3 Small fish models for EDC assessment

Currently, three small-bodied fish models are commonly used for EDC testing by the OECD and US EPA: the fathead minnow, Japanese medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*; Ankley and Johnson, 2004). All three species have short life-cycles, are easily cultured and bred in the lab, and are extensively used in regulatory toxicity testing (Ankley and Johnson, 2004). Whereas the fathead minnow and medaka possess biomarkers that allow for the detection of (anti-)androgenic activity, the zebrafish do not (OECD, 2009). The appearance or reduction in nuptial tubercles in fathead minnow and in papillary processes in medaka are used as biomarkers for (anti-)androgenic chemicals (Ankley and Johnson, 2004). However, in fathead minnows nuptial tubercle alterations are relatively insensitive and not conclusively linked to exposure to (anti-)androgenic chemicals (Katsiadaki and Sebire, 2011). Additionally, a reduction in papillary processes in male medaka is relatively insensitive to anti-androgenic chemical exposure (Katsiadaki and Sebire, 2011; OECD, 2006). Therefore, these biomarkers are not sensitive and/or quantitative enough for the adequate assessment of (anti-)androgenic chemicals.

To address these shortcomings, there have been recent efforts to develop and implement the threespine stickleback (*Gasterosteus aculeatus*) as a bioindicator for (anti-)androgenic chemicals (OECD, 2011).

Threespine stickleback are members of the stickleback family, which includes five genera and 15 other species, that reside in aquatic environments throughout the northern hemisphere (Wootton, 1984). A distinguishing trait of stickleback is the presence of modified dorsal fins as tall sharp spines (Wootton, 1984). Stickleback species are also unique in that males construct nests to house their eggs and fry and are responsible for the parental care of the offspring (Wootton, 1984). Extensive research into nest-building behaviour in threespine stickleback led to the discovery of a nest-building glue named spiggin (Jakobsson et al., 1999). Spiggin is a glycoprotein produced exclusively by reproductively-active male stickleback and used in nest building (Jakobsson et al., 1999). Spiggin is produced in the distal proximal kidney tubules and transported to the urinary bladder, where is it assembled and stored until secretion (Jakobsson et al., 1999). During nest building males secrete spiggin onto nest building material and the highly elastic protein "glues" the nest material together (Jakobsson et al., 1999).

Spiggin protein was eventually explored as a biomarker for (anti-)androgenic chemicals (Katsiadaki et al., 2002b) and is among the most sensitive and quantitative (anti-)androgen-specific biomarkers identified to date in small model fish species (Katsiadaki and Sebire, 2011). In addition, vitellogenin in liver of male threespine stickleback is used as a biomarker for exposure to exogenous estrogenic chemicals (Andersson et al., 2007; Björkblom et al., 2009; Katsiadaki et al., 2010). Other biomarkers in threespine stickleback, used in EDC assessment, include somatic indices and organ histopathology (Andersson et al., 2007; Sanchez et al., 2008b; Wartman et al., 2009).

1.4 EDC biomarkers in threespine stickleback

1.4.1 Spiggin

Spiggin production and the associated hypertrophy of the kidney are androgen-specific responses initiated by the endogenous androgen, 11-ketotestosterone (11-KT; Borg et al., 1993; Jakobsson et al., 1999). De Ruiter and Mein (1982) first identified the secondary proximal tubule epithelium cells in threespine stickleback as the site of production of a mucous substance (later termed spiggin) that was hypothesized to be involved in nest building. They also found that exposure to 11-KT induced the production of mucous within kidney cells, which was accompanied by an increase in cell size and the appearance of secretory granules (De Ruiter and Mein, 1982). Borg et al. (1993) later identified 11-KT as the most potent androgen to induce kidney hypertrophy in castrated male threespine stickleback. Eventually, Jakobsson et al. (1999) was able to extract and characterize the mucous substance in the urinary bladder and nests of male threespine stickleback and named it spiggin. Jakobsson et al. (1999) then exposed castrated males to 11-KT which induced the production of spiggin and simultaneously resulted in kidney hypertrophy. Subsequent characterisation of spiggin identified three subunits (α, β and γ) formed by alternative splicing from a single gene (the spiggin gene) under the control of 11-KT (Jones, 2001; Nagae et al., 2007). Spiggin production was also determined to be restricted to the kidney and urinary bladder (Jones, 2001).

The mechanism underlying spiggin synthesis is thought to be androgen-receptor mediated (Fig. 1.1). Several studies conducted *in vivo* (Hogan et al., 2012; Jolly et al., 2009; Katsiadaki et al., 2006, 2002b; Pottinger et al., 2013) and *in vitro* (Jolly et al., 2009, 2006; Olsson et al., 2005) have demonstrated that spiggin production is stimulated by exposure to androgens whereas co-exposure to anti-androgens suppresses spiggin production. Spiggin is not believed to be induced

through the estrogen receptor (ER), because it is not induced by exposure to steroidal estrogens (Geoghegan et al., 2008; Katsiadaki et al., 2002a). Other chemicals may also alter spiggin production through means other than binding to the AR such as via receptor cross-talk (Jolly et al., 2009) or through non-receptor mediated effects, by acting on circulating steroid levels or receptor expression (Andersson et al., 2007; Hahlbeck, 2004; Katsiadaki and Sebire, 2011). Nevertheless, the degree of induction/reduction of spiggin synthesis exerted through these alternative pathways is minimal relative to the magnitude of response generated by exposure to an androgen (Hahlbeck et al., 2004; Jolly et al., 2006).

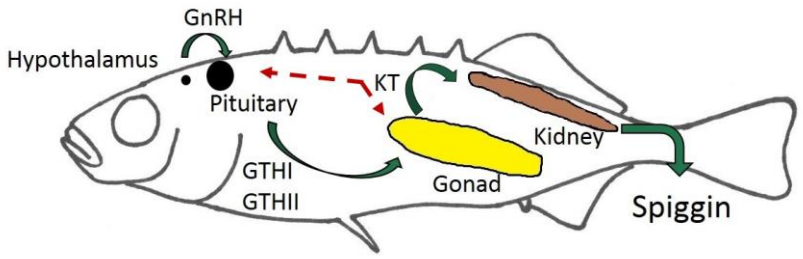

Fig. 1.1 Depiction of the production of the glycoprotein spiggin as regulated by the hypothalamus-pituitary-gonadal axis in male stickleback. Solid green arrows indicate synthesis whereas the red dotted arrows indicate positive and negative feedback on the hypothalamus, pituitary and gonad.

The exploitation of spiggin as a biomarker for androgenic chemical exposure followed a study by Katsiadaki et al. (2002a), which demonstrated that the production of spiggin in females was possible with exposure to androgenic chemicals. Katsiadaki et al. (2002a), developed an enzyme-linked immunosorbent assay (ELISA) to quantify spiggin protein production in the kidney of female and male stickleback exposed to exogenous androgens. The ability to induce spiggin production in females exposed to androgenic chemicals was later confirmed (Allen et al., 2008; Katsiadaki et al., 2002a) and the bioassay was then applied in a field study to evaluate the

(anti-)androgenic activity of WWTPE (Björkblom et al., 2009; Katsiadaki et al., 2012). Assays to measure spiggin mRNA by use of quantitative polymerase chain reaction (qPCR) have been developed (Hogan et al., 2008; Nagae et al., 2007) and used to demonstrate dose-dependent induction of spiggin expression after 7 and 21 days of exposure to an androgen. The study by Hogan et al. (2008) emphasized that measuring spiggin mRNA expression may be a more sensitive method of detecting androgenic chemicals compared to measuring protein expression. The amount of spiggin protein, as measured using an ELISA, increased after exposure to approximately 100 ng/L 17α-methyltestosterone (MT; potent androgen) for 21 days (Katsiadaki et al., 2002a), whereas the abundance of spiggin mRNA, as measured using qPCR, increased after exposure to approximately 10 ng/L MT for 7 days (Hogan et al., 2008). Although a change in mRNA abundance does not necessarily translate into an effect on an organism, the advantage of measuring mRNA over protein expression is that increases in transcript amount may occur at lower exposure concentrations and in a shorter time period compared to protein production.

Spiggin is also measured to assess exposure to anti-androgenic chemicals. The Androgenized Female Stickleback Screening assay (AFSS) in threespine stickleback is the only standardized method available to detect exposure to anti-androgenic chemicals in fish (OECD, 2011). In the AFSS, co-exposure to an androgenic and anti-androgenic chemical is used to induce, and subsequently supress, spiggin production in female threespine stickleback (OECD, 2011). In this way, a threshold for anti-androgenic potency can be established by determining the anti-androgen concentration required to suppress androgen-induced spiggin production (OECD, 2011). Females (as opposed to males) are the test subject because it is difficult to coordinate male reproductive status in order to reduce inter-individual variability in spiggin expression (OECD, 2011). The AFSS has been effective in evaluating the anti-androgenic activity of several

pharmaceuticals and pesticides with reproducible results across different laboratories (Katsiadaki and Sebire, 2011). Anti-androgenicity has also been quantified using a behavioural assay with threespine stickleback whereby reduced nesting and reproductive behaviour in sexually mature males (and reduced spiggin production) indicates exposure to an anti-androgenic chemical (Sebire et al., 2009, 2008). The anti-androgenic activity of several synthetic and environmentally-relevant chemicals and whole-effluents have been evaluated using the male threespine stickleback behavioural assay (Sebire et al., 2011, 2009, 2008).

1.4.2 Vitellogenin

Vitellogenin is an egg-yolk precursor produced in the liver of female oviparous vertebrates in response to increased circulating estrogen (Rotchell and Ostrander, 2003). Oviparous species lay eggs that develop independent from the mother's body (Rotchell and Ostrander, 2003). Vitellogenin is transported from the liver to the ovaries where it forms an egg-yolk precursor which is then deposited into the egg during oogenesis (Rotchell and Ostrander, 2003). Vitellogenin production is essential for embryo survival because yolk is the main food source for fish during early life stages (Rotchell and Ostrander, 2003).

Vitellogenin induction is an ER-mediated process regulated by the HPG axis (Fig. 1.2; Babin et al., 2007). Briefly, GtH1 activates testosterone production by ovarian thecal cells which is aromatized to 17β-estradiol (E2) in granulosa cells (Babin et al., 2007). E2 then binds to an ER in hepatocytes to form a ligand/receptor complex, which then binds to an estrogen response element (ERE) located upstream (or within) of estrogen-responsive genes, initiating vitellogenin

production (Babin et al., 2007). Vitellogenin is then released into the plasma, and ultimately deposited into the oocyte as egg yolk after undergoing proteolytic cleavage (Babin et al., 2007).

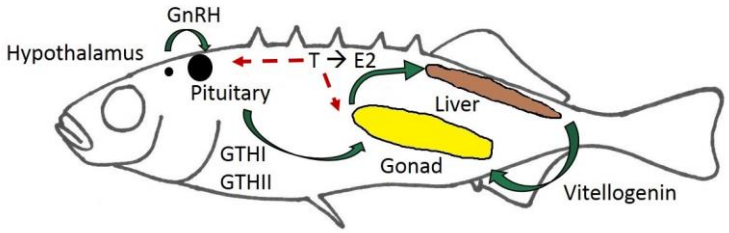

Fig. 1.2 Depiction of vitellogenin production as regulated by the hypothalamus-pituitary-gonadal axis in female stickleback. Solid green arrows indicate synthesis whereas the red dashed arrows indicate positive and negative feedback on the hypothalamus, pituitary and gonad.

Male teleost fish do not typically produce vitellogenin but retain the gene necessary for its synthesis, meaning that vitellogenin production is inducible in males exposed to exogenous estrogens (Rotchell and Ostrander, 2003). Vitellogenin induction in males (and suppression in females) is used extensively as a sensitive biomarker for exposure to (anti-)estrogenic chemicals in fish (Campbell et al., 2006; Goksøyr, 2006; Larkin et al., 2003; Mills and Chichester, 2005; Rotchell and Ostrander, 2003). Two methods have been employed to measure vitellogenin in threespine stickleback. Vitellogenin protein can be measured by ELISA in the heart (Hahlbeck, 2004; Katsiadaki et al., 2010, 2002b) and vitellogenin mRNA can be measured in stickleback liver using qPCR (Hogan et al., 2008). Although these assays exhibit similar sensitivity, the ELISA is not commercially available. The lowest concentration to induce vitellogenin protein in threespine as measured by an ELISA is 18 ng/L following 4 days EE2 exposure (Katsiadaki et al., 2010) whereas the threshold for detection of vitellogenin mRNA after 7 days of E2 exposure is approximately 10 ng/L (Hogan et al., 2008).

There are few studies in the literature investigating vitellogenin as a biomarker for anti-estrogenic chemicals in threespine stickleback. Recently, research into the anti-estrogenic activity of progestins found measurable decreases in vitellogenin in females exposed to 40 ng/L levonorgestrel (Svensson et al., 2013). However, levonorgestrel also induced spiggin production so it was concluded that vitellogenin suppression likely occurred through a non-receptor-mediated pathway (Svensson et al., 2013).

1.4.3 Kidney epithelium cell height

Kidney epithelium cell height (KEH) is a histological biomarker commonly used to assess endocrine disruption in threespine stickleback (Hahlbeck, 2004; Katsiadaki et al., 2002a, 2002b; Wartman et al., 2009). During the breeding season, the kidney in male stickleback hypertrophies in response to spiggin secretion and increasing epithelial cell height in the secondary proximal tubules (Katsiadaki et al., 2002a). Epithelial cell height in the kidney tubules of threespine stickleback increases from approximately 13 µm in reproductively quiescent males to 35 µm during the reproductive season (Katsiadaki et al., 2002a). In females, increased KEH can indicate exposure to exogenous androgens (Katsiadaki et al., 2002a) whereas reduced KEH in androgenized females can indicate exposure to anti-androgenic chemicals (Katsiadaki et al., 2006). Moreover, changes in KEH associated with (anti-)androgenic exposure can be accompanied by increased or decreased spiggin production and kidney hypertrophy or hypotrophy (Katsiadaki et al., 2006; Wartman et al., 2009). KEH is also responsive to complex chemical mixtures; Wartman et al. (2009) found that KEH increased in females exposed to 100% v/v PPME, a response attributed to androgens present in the complex effluent mixture.

The energy demand associated with structural (indicated by KEH) and functional changes in the kidney and the intestine in androgen-exposed stickleback could be detrimental to

the health and survival of the fish in environments contaminated with EDCs. During the developmental transition of the kidney to a reproductive organ, its capacity to excrete water is substantially reduced but, because stickleback require water excretion (in freshwater) to maintain water balance, the intestine adopts the role of osmoregulation leading to increased water output through the anus (De Ruiter et al., 1985; De Ruiter and Mein, 1982). Water excretion by the intestine is accompanied by an increase in mitochondrial area in breeding males and androgen-treated females (De Ruiter et al., 1985) suggesting that this process requires the reallocation of energy. The reallocation of energy towards processes which are not beneficial (e.g. spiggin production and osmoregulation in the intestine in female stickleback) could be detrimental to fish health especially if it reduces the ability to meet metabolic needs elsewhere.

1.4.4 Growth parameters and organosomatic indices

Changes in growth and organosomatic indices (OSI) can indicate exposure (particularly long-term exposure) to EDCs and are considered apical effects that may forecast population-level impacts (Schmitt and Dethloff, 2000). Condition factor (CF) is a common growth parameter and is calculated using Fulton's *K* (where $K = (\text{weight}/\text{length}^3)*100$). Nephrosomatic index (NSI), gonadosomatic index (GSI), and hepatosomatic index (HSI) are OSI that are calculated as (organ weight/total body weight)*100 (Schmitt and Dethloff, 2000).

CF is used to assess fish growth and a low relative CF implies growth suppression that could be caused by contaminants, pathogens, inadequate nutrition or other abiotic factors (Schmitt and Dethloff, 2000). OSI are affected by various factors (Schmitt and Dethloff, 2000). For example, under normal conditions HSI is 2% of the total body weight of the fish whereas if HSI is higher than 2% it is generally a sign of altered metabolism or energy reserves (Schmitt and Dethloff, 2000). High HSI can also indicate effects from contaminant exposure because

contaminants are actively metabolized in the liver (Andersson et al., 2007). Additionally, during vitellogenin production in female fish the liver increases in size and so, increased HSI in males may be a result of vitellogenin production (Andersson et al., 2007; Schmitt and Dethloff, 2000). There are several other factors to consider in evaluating HSI including inter- and intra-specific species differences, reproductive state, season, diet, and stress (Schmitt and Dethloff, 2000).

Another useful tool to assess EDCs is GSI, calculated as the relative weight of the gonad as a percent value of total body weight (Schmitt and Dethloff, 2000). Exposure to an EDC can result in abnormal gonadal development thereby altering GSI (Schmitt and Dethloff, 2000). Several studies have reported no change in GSI in threespine stickleback exposed to androgenic, and estrogenic chemicals or sewage effluent (Allen et al., 2008; Björkblom et al., 2009; Sanchez et al., 2008b), which suggests that GSI is not a reliable indicator to evaluate EDC exposure and effects in this species. Although unresponsive to EDC exposure in threespine stickleback, evaluating GSI is inexpensive, relatively easy to perform, and has been demonstrated as an effective biomarker in several other fish species (Schmitt and Dethloff, 2000). Factors including the reproductive stage of individual fish, within-species variability (e.g., hierarchy, nutrition) and interspecific differences may affect GSI and should be considered in its interpretation (Schmitt and Dethloff, 2000).

Dramatic hormone-dependent structural changes occur in the male kidney throughout the reproductive season (Sokołowska and Kulczykowska, 2006). An increase in male NSI is attributed to kidney hypertrophy prior to the breeding season in response to an increase in circulating endogenous androgen levels (Borg et al., 1993). NSI naturally fluctuates in male stickleback according to the reproductive cycle and is low in mature male stickleback from late

August to September at which point it begins to progressively increase to its peak in May (Sokołowska and Kulczykowska, 2006).

Changes in NSI are used as a robust indicator of exposure to exogenous estrogenic or androgenic chemicals in threespine stickleback. Exogenous estrogens can reduce NSI by interfering with normal kidney growth or by inhibiting spiggin production and thereby reducing kidney weight. Andersson et al. (2007) reported reduced NSI in male threespine stickleback exposed to EE2 and attributed this response to inhibited kidney growth or supressed spiggin production. Female kidneys can also undergo hypertrophy as a result of exposure to exogenous androgens or to complex effluent mixtures containing androgenic chemicals (Katsiadaki et al., 2002a; Wartman et al., 2009). An increase in NSI is associated with spiggin production and is therefore, an androgen-dependent response; Björkblom et al. (2009) measured a significant positive correlation between NSI and spiggin production in MT-exposed males and females. A decrease in KEH has also been reported in androgenized females with co-exposure to flutamide (FL: potent anti-androgen; Katsiadaki et al., 2006) and, although changes in NSI have not been measured with exposure to anti-androgenic chemicals it is likely that kidney hypotrophy accompanies a decrease in KEH. Since endogenous androgen levels may interfere with the response of this endpoint in male stickleback, it is a more robust endpoint for EDC exposure in females (Katsiadaki et al., 2006)

1.5 *Culaea inconstans* as a bioindicator for EDCs

The broad distribution, small size (< 87 mm in length) and hardiness of brook stickleback (*Culaea inconstans*) support its use as an indicator species. Within Canada, brook stickleback are abundant in their native range from Rocky Mountains in the west, to Great Slave Lake in the north, northeast to Hudson Bay, and east to the Great Lakes and the St. Lawrence estuary

(Wootton, 1984). They are located in a multitude of habitats including lakes, ponds, ephemeral and permanent streams, rivers, bogs, seasonal meltwater, potholes, sinkholes, hotspring lakes, ditches, and beaver ponds (Stewart et al., 2007). In milder seasons, they are found in slow moving waters near shorelines, or in shallower waters with dense vegetation and soft substrates (Stewart et al., 2007). In winter they will migrate into deeper, warmer waters and are considered a relatively sedentary fish (Wootton, 1984). The impact of confounding factors in ecological risk assessment is lessened when a fish has a small home range because exposure is more likely to have occurred in the area of capture (Pottinger, 2002). They are also able to survive deoxygenated conditions and water temperatures as low as -2 °C (Reisman and Cade, 1967; Wootton, 1984). They have long been used as a laboratory fish species and can be successfully acclimated to lab conditions with little or no mortality (McKenzie, 1969; Reisman and Cade, 1967). Moreover, the reproductive state of sticklebacks can be equally initiated or suppressed by altering photoperiod and water temperature (Katsiadaki et al., 2002a; Sebire and Katsiadaki, 2008). Although this has not been specifically described in brook stickleback it has been found to be true for its coastal relative, the threespine stickleback (Katsiadaki et al., 2002a; Sebire and Katsiadaki, 2008). The distribution of brook stickleback throughout freshwaters in interior Canada also makes them an ecologically relevant species with which to assess EDC exposure. In North America, threespine stickleback reside in coastal and estuarine environments whereas brook stickleback inhabit fresh and brackish water (Stewart et al., 2007; Wootton, 1984). Therefore, EDC assessment in the field using threespine stickleback is limited to coastal and estuarine environments. However, both species could serve as bioindicators for EDCs (threespine in coastal environments and brook in freshwater environments) provided that the

endpoints measured in threespine stickleback for EDC assessment are also measurable in brook stickleback.

1.6 Project rationale and objectives

(Anti-)androgenic compounds have been detected in freshwaters from inputs such as MWWE, industrial outflows and agricultural runoff - yet model fish species commonly used in exposure studies lack sensitive and quantifiable biomarkers for evaluating exposure to such compounds. Threespine stickleback have emerged as a bioindicator species for (anti-)androgenic EDCs using the androgen-responsive biomarker spiggin; however, their habitat is limited to coastal and estuarine environments in North America and so, they cannot be used in the biomonitoring of EDCs in freshwater environments. Alternatively, their close relative, the brook stickleback, are ubiquitous throughout central Canada, can be easily captured in a multitude of environments, and can be maintained in a laboratory. Although brook stickleback have been used in fish biology and environmental monitoring studies, no studies to date have characterised and evaluated potential biomarker responses to hormone exposure in this species. Such an initiative would greatly enhance the usefulness of brook stickleback in laboratory bioassays for EDCs and potentially as a biomonitoring species in areas of EDC contamination in the field. Therefore, the overall objective of this thesis was to determine if brook stickleback are a suitable bioindicator species for EDCs by evaluating their response and sensitivity to estrogenic and (anti-)androgenic chemicals. The specific objectives and associated hypotheses are as follows:

1. To quantify basal spiggin and vitellogenin mRNA abundance in wild-caught adult female and male brook stickleback (Chapter 2).

H_o: Spiggin and vitellogenin mRNA are not differentially expressed in adult male and female brook stickleback.

2. To determine the sensitivity of brook stickleback exposed to model androgenic and estrogenic chemicals using traditional and novel biomarkers for endocrine disruption (Chapter 2)

   H_o: There are no differences in spiggin mRNA expression, condition factor, organosomatic indices or kidney epithelium cell height between control fish and fish treated with 17α-methyltestosterone.

   H_o: There are no differences in vitellogenin mRNA expression, condition factor, organosomatic indices or kidney epithelium cell height between control fish and fish treated with 17α-ethinylestradiol.

3. To determine the sensitivity of androgenized female brook stickleback exposed to a model anti-androgen using traditional and novel biomarkers for endocrine disruption (Chapter 3).

   H_o: There are no differences in spiggin mRNA expression, condition factor, organosomatic indices or kidney epithelium cell height among control and brook stickleback co-exposed to 17α-methyltestosterone and flutamide.

CHAPTER 2

2. BIOMARKER RESPONSES TO ESTROGEN AND ANDROGEN EXPOSURE IN BROOK STICKLEBACK (CULAEA INCONSTANS): A NEW BIOINDICATOR SPECIES FOR ENDOCRINE DISRUPTING COMPOUNDS[1]

[1]This chapter is in press in Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology (2016) 180, 1 – 10, under joint authorship with Dr. N. Hogan (University of Saskatchewan). B. Muldoon designed and conducted the experiments, collected and analyzed the data and wrote the manuscript. N. Hogan supervised B. Muldoon, provided support throughout the exposure, and assisted in the preparation of the manuscript. The tables, figures and references cited herein have been reformatted to adhere to the thesis style. References for this chapter can be found in the reference section for this thesis.

2.1 Introduction

There is ongoing concern surrounding the release of compounds into the aquatic environment due to their potential to disrupt the endocrine system of many aquatic species. Exposure to endocrine disrupting compounds (EDCs) can induce changes which span multiple levels of biological organization from molecular to whole-organism, including (but not limited to) alterations in transcript and protein levels, changes in concentrations of circulating sex steroids, histopathological changes in target tissues, delayed or altered development, reproductive failure and abnormal behaviour in fish (reviewed in Arukwe, 2001; Hutchinson et al., 2006; Rotchell and Ostrander, 2003). More recently, exposure to EDCs has been implicated in population-level effects, including the collapse and recovery of fish populations (Blanchfield et al., 2015; Kidd et al., 2007). To determine effect thresholds for EDCs several small fish have been applied as model species in regulatory testing through the assessment of biomarker responses which range from mechanistic endpoints to ecologically relevant effects (Ankley and Johnson, 2004; Scholz and Mayer, 2008).

In North America, the fathead minnow (*Pimephales promelas*) is the most common small model fish species used to assess estrogenic and androgenic activity of chemicals (Ankley and Johnson, 2004). Several endpoints in this species are responsive to estrogens, including increased expression of vitellogenin, altered sex-steroid production, decreased nuptial tubercle counts in males, delayed gonadal maturation and decreased fecundity and fertility (Bringolf et al., 2004; Pawlowski et al., 2004b; Seki et al., 2006). Similarly, assays of several secondary sex characteristics in the fathead minnow have been developed as biomarkers of exposure to androgenic compounds, including nuptial tubule growth, changes in the size of the dorsal nape pad and alterations in shape, colouration and breeding behaviour (Ankley et al., 2003;

Martinović et al., 2008; Pawlowski et al., 2004a). However, these biomarkers are often not responsive to low concentrations of androgenic compounds and are scored using a subjective scale; thus, they may be influenced by measurement bias (Ankley et al., 2003; Martinović et al., 2008; Pawlowski et al., 2004a). Secondary sex characteristics can also be affected by the hierarchical status of the fish (Martinović et al., 2008) making it difficult to attribute a change in appearance to exposure to an EDC. Although the fathead minnow possess unique hormone-responsive traits, there are limitations to using this species for screening and testing of androgenic compounds.

The threespine stickleback (*Gasterosteus aculeatus*) is a small-bodied model fish species that has been more recently used in bioassays to assess the endocrine activity of compounds. Male stickleback produce a unique, quantifiable and androgen-responsive glycoprotein in the kidney called spiggin, which is used to construct nests during the breeding season (Jakobsson et al., 1999). Although female sticklebacks do not normally produce spiggin, exposure to exogenous androgens will induce spiggin production in females, which can be measured in the form of transcript or protein expression (Hogan et al., 2008; Katsiadaki et al., 2002a, 2002b). Spiggin production occurs in the proximal tubules of the kidney, so increased kidney weight and kidney epithelium cell height (KEH) have also been used as biomarkers of androgen exposure (Katsiadaki et al., 2002a). Finally, vitellogenin production by the liver in males can also be assessed as an indicator of estrogen exposure, making threespine stickleback one of the few fish species with biochemical and apical endpoints for both (anti-)androgens and (anti-)estrogens. However, the geographical distribution of this species is mostly limited to coastal and estuarine environments in North America and Europe, which restricts their application as a monitoring species in freshwater systems.

Brook stickleback (*Culaea inconstans*) could potentially be used as an additional small fish model, to the threespine stickleback, for regulatory testing and ecological monitoring of EDCs. This species has similar reproductive behaviours and physiology as the threespine (Stewart et al., 2007) and is therefore hypothesized to have similar measurable responses to exogenous hormone exposure. Brook stickleback are widely distributed in freshwater systems in North America (Wootton, 1984) and have been previously studied for reproductive behaviour (McKenzie, 1969; McLennan, 1993; Reisman and Cade, 1967). Recently, brook stickleback were used as a sentinel species to evaluate the effects of municipal wastewater effluents in an effluent-dominated stream in Saskatchewan, Canada (Tetreault et al., 2012). Therefore, the objective of this study was to develop a bioassay using brook stickleback to measure estrogenic and androgenic responses and determine the sensitivity of various traditional and novel biomarkers of exposure. To achieve this, we first developed and validated a real-time qPCR method to assess the expression of spiggin in the kidney and vitellogenin in the liver of brook stickleback. Sex differences in basal transcript abundance were assessed using reproductively mature males and females. A short-term exposure to a 17α-methyltestosterone and 17α-ethinylestradiol (ng/L range) was employed to determine the hormonal responsiveness of these transcripts in addition to apical endpoints such as organosomatic indices, condition factor and histopathological changes in kidney tissues. Sampling was conducted after 7 and 21 days of exposure to determine the response of these biomarkers over time.

2.2 Material and methods

2.2.1 Animals

Brook stickleback used in this study were collected from the Cranberry Flats Conservation Area near Saskatoon, Saskatchewan, Canada. Cranberry Flats is a marshy wetland that connects to the South Saskatchewan River during the spring snowmelt runoff season. All fish were collected with dip nets. Fish used to assess basal transcript levels of spiggin and vitellogenin in males versus females were collected in mid-breeding season in July 2014 while stickleback used for the steroid hormone exposures were collected during September 2013. Fish were transported to the Aquatic Toxicology Research Facility (Toxicology Centre, University of Saskatchewan, Canada) and placed in an 84" x 24" x 14.5" Min-O-Cool supplied with a constant flow of filtered facility water. Animals were maintained at a temperature of 16 ± 1 °C with a photoperiod of 12:12 (light:dark). Fish were fed twice daily with frozen blood worms (Sally's Bloodworms, San Francisco Bay Brand, CA, United States). All methods used in the present study were approved by the University of Saskatchewan's Animal Research Ethics Board (AUP #: 20130105) and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

2.2.2 Laboratory exposures and sampling

The synthetic steroids, 17α-methyltestosterone (MT; ≥ 98% purity) and 17α-ethinylestradiol (EE2; ≥ 98% purity), used for fish exposures were purchased from Sigma-Aldrich (Oakville, ON, Canada). Male and female brook stickleback were transferred from a holding tank to 10-gallon glass tanks one week prior to exposure with a fish loading rate in compliance with OECD recommendations for the 21 day fish assay at 0.5 g fish/ litre water

(OECD, 2009)MT: 20 fish/tank; EE2: 18 fish/tank). Fish were exposed to three nominal concentrations (1, 10 and 100 ng/L) of either MT or EE2, or to an acetone carrier control (0.002%; henceforth referred to as control). Exposures were conducted in a static-renewal system with two-third water renewal and re-dose of chemical every 48 hours. Previous exposures to MT using a similar semi-static exposure regime have elicited androgenic responses in the threespine stickleback and measurement of MT in water revealed actual concentrations that were approximately 80% of nominal (Katsiadaki et al., 2006, 2002a). All treatments were run in triplicate. Water temperature was maintained at 16 ± 2 °C, photoperiod at 16:8 (light:dark) and water quality measurements were measured each week using API Aquarium test strips (pH: 7.5 – 8.4; ammonia: 0.25 – 0.5 mg/L).

Sampling was conducted on day 7 and 21 with half of the fish randomly sampled each day (MT: 30 fish/treatment/day; EE2: 27 fish/treatment/day). Fish were stunned with a blow to the head, weighed and total fork length measured (to the nearest 0.1 mm) prior to being killed by spinal severance. The liver, kidney and gonad were excised, weighed (to the nearest 0.01 g), immediately flash frozen on dry ice and stored at -80 °C until RNA extraction. A subset of samples (4-9 fish/treatment/day) were collected from one tank in each exposure group for kidney histology on day 7 and 21 (MT exposure) and day 21 (EE2 exposure). Fish selected for kidney histology were placed whole (incision made in the abdomen) into 10% neutral-buffered formalin and later transferred to 70% ethanol for long-term storage prior to processing.

2.2.3 RNA extraction and reverse transcription

Total RNA was obtained from kidney and liver using E.Z.N.A. MicroElute Total RNA Kit following manufacturer protocols (Omega BioTek, Norcross, GA, United States). RNA was treated with DNase to eliminate gDNA contamination using Turbo DNA-free kit (Ambion,

Burlington, ON, Canada). RNA concentration was quantified on a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, United States) and quality was verified by gel electrophoresis on a 1% tris-borate EDTA agarose gel. Reverse transcription was performed with an RNA input of 1 µg for liver and 0.25 µg for kidney using iScript cDNA synthesis kit according to the manufacturer (Bio-Rad Laboratories Inc., Hercules, CA, United States). cDNA was diluted at 10- and 100-fold for liver and 25-fold for kidney for subsequent gene expression analysis.

2.3.4 Primer design, optimization and real-time PCR analysis of spiggin and vitellogenin

Spiggin and vitellogenin primers for qPCR were designed from partial sequences amplified using degenerate primers. Degenerate primers were designed against previously identified sequences from other fish species obtained from GenBank (http://www.ncbi.nlm.nih.gov/ Genbank/index.html) (spiggin: AF323733, DQ018715; vitellogenin: EU399547.1, AB181839.1, DQ0202121). Sequences were aligned using Geneious Pro 5.6.5 (BioMatters) and primers were designed on Geneious Pro from the consensus sequence for spiggin (sense primer 5'- TACGA(CT)CACACCTACCAGCA – 3'; antisense primer 5' TCACAAA(GC)TGGCCTTCAATG-3') and vitellogenin (sense primer 5'-(AC)AC(CT)TCAGCCCTGGCTGCTC-3'; antisense primer 5'-CCATCTG(AG)GCAGC(AG)CCATTC-3') and purchased from Invitrogen (Life Technologies Inc., Burlington, ON, Canada). PCR reactions of 25 µL were conducted with TopTaq DNA Polymerase kit (Qiagen, Mississauga, ON, Canada). The PCR mixture consisted of 1x TopTaq PCR Buffer, 0.2 mM of forward and reverse primer, 1 µL of cDNA template and 11 µL of RNase-free water. PCR amplification was performed on Eppendorf Mastercycler Gradient Thermal Cycler under the following conditions: initial denaturation for 3 min (94 °C) followed

by 40 cycles of denaturation for 30 s (94 °C), annealing for 30 s (60 °C) and extension for 1 min (72 °C). After 40 cycles the reaction ended with a final extension for 10 min (72 °C). The amplification product was then run on a 1% agarose gel and the DNA product was excised and purified using Qiagen's QIAEX II Gel Extraction Kit. The extracted product was submitted for sequencing at Plant Biotechnology Institute of Canada (National Research Council, Saskatoon, Canada). The resulting partial sequence for spiggin (651 bp) was 87-91% similar to that of threespine stickleback spiggin alpha and beta whereas the partial sequence for vitellogenin (424 bp) was 91% similar to that of threespine stickleback vitellogenin.

Basal and steroid hormone-induced transcript levels were measured using quantitative polymerase chain reaction (qPCR) system on a CFX96 Real-time C1000 Thermal Cycler (Bio-Rad). Partial sequences obtained as described above were used as the template to design gene specific primers for amplification of spiggin in the kidney and vitellogenin in the liver whereas primers for the two reference genes, 18S and ribosomal protein L8 (RPL-8), were sourced from the literature (Table 2.1). Primer sets were optimized to determine cDNA input for minimal C_t, optimal annealing temperature and a single sequence-specific peak in the melt curve using SYBR Green based detection systems (data not shown). PCR reactions were run as 20 µL volume with 1x SsoFast EvaGreen Supermix (Bio-Rad), 0.4 mM each of forward and reverse primers (Invitrogen) and 2 µL of diluted cDNA template. The following conditions were used for amplification: initial denaturation for 30 sec at 95 °C, followed by 40 cycles of 5 s at 95 °C, 5 s at annealing temperature (Table 2.1); 95 °C for 1 min; and then melt curve analysis starting at 55 °C and increasing in 1 °C increments to 95 °C every 30 s. Each plate contained no-template controls and no-reverse transcriptase controls as negative controls.

Table 2.1 Primers for real-time PCR analysis of gene expression in brook stickleback (*Culaea inconstans*).

Target	Primer	Sequence (5’ to 3’)	Amplicon length (bp)	Annealing Temp (°C)	Reference
Spiggin	Forward	TTCGGAAAACCAAGAACTGTCT	227	60	this paper
	Reverse	ATGCTGGACCCTTTTTCTCATA			
Vitellogenin	Forward	TGTGTGCAAGACCCACTATCTC	153	60	this paper
	Reverse	TCCTCTAGCCTCACACTCAACA			
18S	Forward	GGCGGCGTTATTCCCATGACC	110	62	Esbaugh et al., 2008
	Reverse	GGTGGTGCCCTTCCGTCAATTC			
RPL-8	Forward	CGACCCGTACCGCTTCAAGAA	143	60	Shao et al., 2015
	Reverse	GGACATTGCCAATGTTCAGCTGA			

In addition to melt-curve analysis, standard curves for each primer set were constructed using serially diluted cDNA template from representative samples. Linear curves produced for each primer set had efficiencies of 100 - 110% and $R^2 \geq 0.98$. Standard curves were run on each plate (accepted only if efficiency = 90 – 110%, $R^2 \geq 0.98$) and the relative standard curve method (Applied Biosystems, 1997) was used to determine the relative transcript abundance (all samples assayed in duplicate). To correct for minor variations in template input and transcriptional efficiency between samples, relative abundance for spiggin in the kidney and vitellogenin in the liver was normalized against the expression values of the reference genes, 18S and RPL-8, in their respective tissues. The geometric mean of these two reference genes was used for normalization.

2.2.5 Condition factor and organosomatic indices

Condition factor (CF) was calculated using Fulton's *K* (Ricker, 1975). The organosomatic indices, including hepatosomatic index (HSI), gonadosomatic index (GSI), and nephrosomatic index (NSI) were calculated by dividing liver, gonad or kidney weight (respectively) by the weight of the fish and multiplying by 100.

2.2.6 Kidney Histology

Preserved fish were sectioned posterior to the pectoral fin and at the anus prior to tissue processing. Tissues were processed through step-wise dehydration in ethanol, cleared with xylene and embedded in paraffin. Sections were cut at 5 µm thickness with nine transverse sections prepared each from posterior, middle and anterior portion of the abdomen which were approximately 50 µm apart in order to ensure the presence of many secondary proximal tubules of the kidney tissue. Sections were stained with hematoxylin-eosin. KEH was measured using

AxioVision Rel. 4.8 Software (Carl Zeiss Canada, Toronto) on three serial sections from posterior, middle and anterior portion of the kidney on three tubules per section with three measurements conducted on each tubule. Mean KEH from individual fish were averaged to obtain KEH for each treatment. KEH was analyzed using a masked (or blind) evaluation and the results were verified by a second blinded assessment of a subsample of kidneys with the values verified against original data.

2.2.7 Statistical analysis

Data were tested for normality using Kolmogorov-Smirnov test on residuals and for homogeneity of variance using Levene's test. Data not meeting the assumptions of parametric statistics were log transformed prior to being re-analysed. The differential expression of basal spiggin and vitellogenin in male versus female wild-caught brook stickleback was analyzed using a student's t-test. Hormone-induced (EE2 and MT) data for organosomatic indices and CF were analyzed separately in males and females using a nested ANOVA, with treatment and tank as factors in the hierarchical order listed, and the nest factor (tank) was removed if p-value was ≥ 0.250. Data was then re-run as a one-way ANOVA. Hormone-induced (MT and EE2) gene expression data were analyzed using a one-way ANOVA with spiggin analyzed in the kidney of MT-exposed females and vitellogenin in the liver of EE2-exposed males. The expression of spiggin in the kidney of control males in the MT trial and vitellogenin expression in the liver of control females in the EE2 trial were also measured to determine baseline expression in unexposed fish but this data were not included in the statistical analysis. A post-hoc Dunnett's test for OSI, CF and gene expression data in hormone-treated fish compared each treatment to the solvent-control. All analysis were conducted on IBM SPSS Statistics 22 (Armonk, NY, United States).

2.3 Results

2.3.1. Sex difference in basal spiggin and vitellogenin transcript levels

Wild-caught mature brook stickleback exhibited differences in spiggin and vitellogenin transcript levels between sexes (Fig. 2.1). Spiggin mRNA was significantly higher (17-fold) in the kidney of male stickleback relative to the female ($p = 0.001$, Fig. 2.1A). Alternately, vitellogenin mRNA was significantly higher (77-fold) in the liver of female stickleback relative to the male ($p = 0.001$, Fig. 2.1B).

2.3.2 Effects of MT/EE2 on length, weight, condition factor and organosomatic indices

A significant increase in CF was observed in fish exposed to 1 ng/L MT after 7 d ($p = 0.015$, Table 2.2). There was no significant difference in CF at any other MT concentration or sampling day relative to control and, as an isolated event, the change observed in CF is unlikely to be biologically significant. There was also no significant difference in length, weight, GSI, HSI or NSI at any concentration of MT relative to the control after either 7 or 21 d (Table 2.2).

There was no significant difference in length, weight or CF at any concentration of EE2 relative to control after 7 or 21 d (Table 2.3). A significant reduction in GSI occurred in females exposed to 1 ng/L ($p = 0.006$), 10 ng/L ($p = 0.001$) and 100 ng/L ($p = 0.001$) EE2 relative to control after 21 days (Table 2.3). There was no significant difference in male GSI at any concentration of EE2 relative to control. A significant increase in HSI occurred in the 100 ng/L EE2 treatment in females but not males after 7 d ($p = 0.043$) and in both females ($p = 0.012$) and males ($p = 0.007$) after 21 d (Table 2.3). NSI did not significantly differ between EE2 treatments and control in males or females at any concentration tested (Table 2.3)

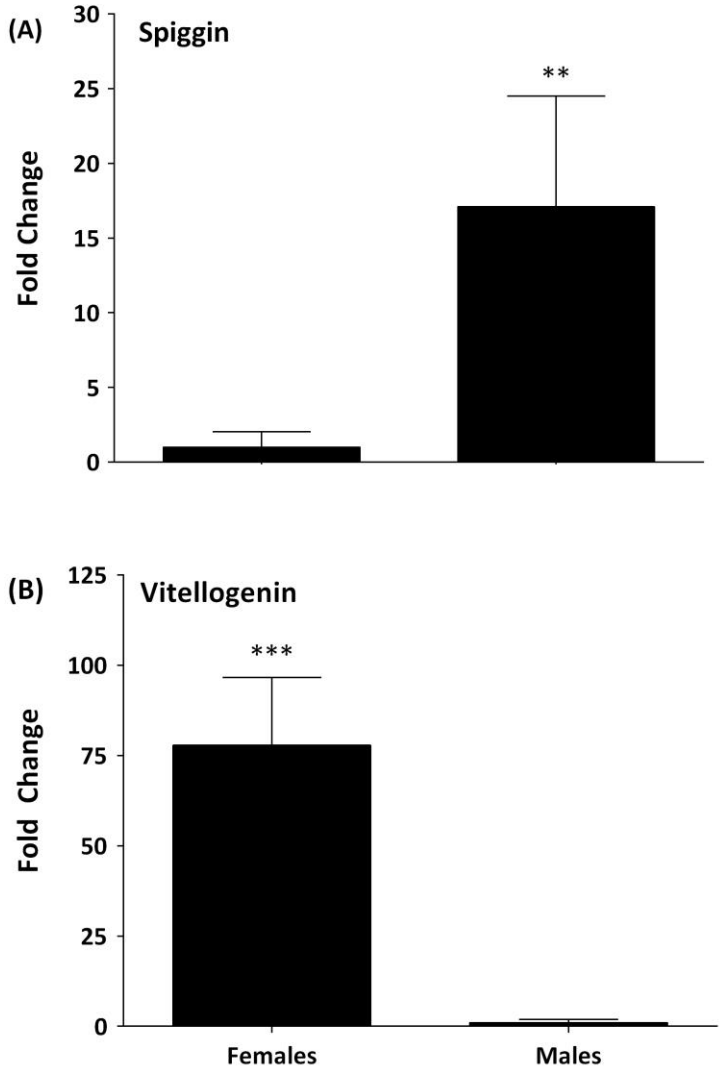

Fig. 2.1 Differential expression of (A) spiggin and (B) vitellogenin in adult male versus female brook stickleback (*Culaea inconstans*) measured using real-time qPCR. Spiggin in the kidney and vitellogenin in the liver was measured in experimental groups of 6-8 fish with each sample analyzed in duplicate. Data are shown as relative fold difference (mean ± SEM) between males and females. A student's t-test was used to compare males and females with significant differences indicated by an asterisk (**$p < 0.01$, ***$p < 0.001$).

Table 2.2 Mean (± SE) length, weight, hepatosomatic indices (HSI), gonadosomatic indices (GSI), nephrosomatic index (NSI) and condition factor (CF) of male and female brook stickleback (*Culaea inconstans*) exposed to 17α-methyltestosterone (MT) for 7 and 21 days. Treatments are MT 1 ng/L (MT 1), 10 ng/L (MT 10) and 100 ng/L (MT 100).

Sex (treatment duration)	Variable	Control	MT 1	MT 10	MT 100
Female (7 day)	**Length (mm)**	3.8 ± 0.1	4.1 ± 0.2	4.0 ± 0.1	3.6 ± 0.1
	Weight (g)	0.41 ± 0.03	0.52 ± 0.07	0.46 ± 0.03	0.35 ± 0.03
	HSI (%)	3.0 ± 0.4	3.0 ± 0.4	2.2 ± 0.3	2.6 ± 0.3
	GSI (%)	4.1 ± 0.7	5.1 ± 0.5	5.0 ± 0.6	4.2 ± 0.3
	NSI (%)	0.30 ± 0.08	0.26 ± 0.06	0.26 ± 0.07	0.36 ± 0.08
	CF	0.73 ± 0.02	0.74 ± 0.02	0.70 ± 0.02	0.73 ± 0.02
Female (21 day)	**Length (mm)**	3.9 ± 0.1	4.0 ± 0.1	3.8 ± 0.2	4.0 ± 0.1
	Weight (g)	0.42 ± 0.03	0.41 ± 0.02	0.42 ± 0.02	0.43 ± 0.02
	HSI (%)	3.3 ± 0.2	3.3 ± 0.3	3.1 ± 0.3	2.6 ± 0.2
	GSI (%)	3.3 ± 0.6	3.3 ± 0.3	4.2 ± 1.0	3.6 ± 0.5
	NSI (%)	0.29 ± 0.1	0.23 ± 0.03	0.17 ± 0.03	0.31 ± 0.05
	CF	0.70 ± 0.01	0.72 ± 0.03	0.69 ± 0.02	0.64 ± 0.02
Male (7 day)	**Length (mm)**	4.0 ± 0.1	3.7 ± 0.1	3.8 ± 0.1	4.0 ± 0.1
	Weight (g)	0.44 ± 0.04	0.39 ± 0.03	0.41 ± 0.05	0.47 ± 0.04
	HSI (%)	2.6 ± 0.2	2.8 ± 0.2	2.7 ± 0.3	2.6 ± 0.1
	GSI (%)	0.80 ± 0.08	0.69 ± 0.08	0.80 ± 0.11	0.77 ± 0.14
	NSI (%)	0.49 ± 0.09	0.52 ± 0.06	0.29 ± 0.12	0.36 ± 0.12
	CF	0.69 ± 0.02	0.75 ± 0.02*	0.69 ± 0.02	0.73 ± 0.01
Male (21 day)	**Length (mm)**	4.0 ± 0.1	4.0 ± 0.1	3.9 ± 0.1	3.7 ± 0.1
	Weight (g)	0.44 ± 0.03	0.45 ± 0.03	0.44 ± 0.03	0.41 ± 0.03
	HSI (%)	3.1 ± 0.18	3.6 ± 0.28	3.1 ± 0.19	3.0 ± 0.20
	GSI (%)	0.80 ± 0.13	0.73 ± 0.10	0.65 ± 0.08	0.76 ± 0.09
	NSI (%)	0.33 ± 0.05	0.28 ± 0.06	0.27 ± 0.03	0.26 ± 0.09
	CF	0.70 ± 0.02	0.73 ± 0.03	0.71 ± 0.02	0.73 ± 0.02

Table 2.3 Mean (± SE) length, weight, hepatosomatic indices (HSI), gonadosomatic indices (GSI), nephrosomatic index (NSI) and condition factor (CF) of male and female brook stickleback (*Culaea inconstans*) exposed to 17α-ethinylestradiol (EE2) for 7 and 21 days. Treatments are EE2 1 ng/L (EE2 1), 10 ng/L (EE2 10) and 100 ng/L (EE2 100).

Sex (treatment duration)	Variable	Control	EE2 1	EE2 10	EE2 100
Female (7 day)	**Length (mm)**	4.1 ± 0.1	4.1 ± 0.1	4.0 ± 0.1	4.3 ± 0.1
	Weight (g)	0.48 ± 0.03	0.51 ± 0.04	0.47 ± 0.03	0.53 ± 0.03
	HSI (%)	3.5 ± 0.16	4.1 ± 0.37	3.9 ± 0.35	5.0 ± 0.50*
	GSI (%)	1.5 ± 0.09	1.5 ± 0.13	1.5 ± 0.19	1.3 ± 0.14
	NSI (%)	0.17 ± 0.02	0.13 ± 0.02	0.13 ± 0.02	0.11 ± 0.001
	CF	0.71 ± 0.02	0.72 ± 0.02	0.72 ± 0.02	0.66 ± 0.02
Female (21 day)	**Length (mm)**	4.2 ± 0.1	4.2 ± 0.1	4.1 ± 0.1	4.1 ± 0.1
	Weight (g)	0.51 ± 0.04	0.52 ± 0.03	0.49 ± 0.03	0.50 ± 0.02
	HSI (%)	3.4 ± 0.2	3.7 ± 0.3	3.7 ± 0.3	4.7 ± 0.3*
	GSI (%)	2.1 ± 0.1	1.6 ± 0.1**	1.6 ± 0.1***	1.4 ± 0.1***
	NSI (%)	0.12 ± 0.02	0.12 ± 0.03	0.110± 0.01	0.11 ± 0.02
	CF	0.70 ± 0.03	0.70 ± 0.02	0.67 ± 0.02	0.71 ± 0.02
Male (7 day)	**Length (mm)**	4.2 ± 0.1	4.1 ± 0.1	4.2 ± 0.1	4.2 ± 0.1
	Weight (g)	0.55 ± 0.03	0.55 ± 0.03	0.55 ± 0.03	0.53 ± 0.05
	HSI (%)	4.0 ± 0.4	4.3 ± 0.3	4.4 ± 0.4	4.0 ± 0.5
	GSI (%)	1.1 ± 0.1	1.0 ± 0.1	1.4 ± 0.2	0.84 ± 0.13
	NSI (%)	0.11 ± 0.02	0.11 ± 0.01	0.17 ± 0.03	0.12 ± 0.02
	CF	0.71 ± 0.02	0.77 ± 0.02	0.69 ± 0.07	0.71 ± 0.02
Male (21 day)	**Length (mm)**	4.2 ± 0.1	4.2 ± 0.1	4.4 ± 0.1	4.3 ± 0.1
	Weight (g)	0.51 ± 0.04	0.54 ± 0.05	0.60 ± 0.04	0.56 ± 0.03
	HSI (%)	3.4 ± 0.3	3.7 ± 0.4	3.6 ± 0.2	4.8 ± 0.2**
	GSI (%)	1.4 ± 0.1	1.0 ± 0.1	1.2 ± 0.2	1.0 ± 0.1
	NSI (%)	0.15 ± 0.02	0.11 ± 0.02	0.08 ± 0.01	0.11 ± 0.01
	CF	0.70 ± 0.02	0.71 ± 0.02	0.71 ± 0.02	0.70 ± 0.02

2.3.3 Effects of MT/EE2 on spiggin and vitellogenin transcript levels

Spiggin mRNA in the kidney of female fish was significantly increased in the 100 ng/L MT treatment relative to control by more than three orders of magnitude after 7 d ($p = 0.001$, Fig. 2.2A) and more than two orders of magnitude after 21 d ($p = 0.001$, Fig. 2.2B). There was no significant difference in spiggin mRNA in the kidney of female fish exposed to 1 ng/L MT relative to control after 7 d (1.5-fold; $p = 0.878$) or 21 d (1.7-fold; $p = 0.849$). Similarly, there was no significant difference in spiggin mRNA in the kidney of female fish exposed to 10 ng/L MT relative to control after 7 d (3-fold; $p = 0.302$) or 21 d (7-fold; $p = 0.055$). Spiggin mRNA in males exposed to MT was not quantified. Vitellogenin was not significantly upregulated in the 100 ng/L MT treatment relative to control in the liver of male stickleback after 7 d ($p = 0.703$, data not shown). Vitellogenin was not measured at any other MT exposure concentration or after 21 days of exposure to MT because mRNA induction did not occur after 7 days of exposure to the highest tested MT concentration (100 ng/L).

Vitellogenin mRNA in the liver of male fish was significantly increased in the 100 ng/L EE2 treatment relative to control by more than five orders of magnitude after 7 d ($p = 0.001$, Fig. 2.3A) and 21 d ($p = 0.001$, Fig. 2.3B). There was no significant difference in vitellogenin mRNA in the liver of male fish after 7 d exposure to EE2 at 1 ng/L (1.1-fold; $p = 0.999$) and 10 ng/L (2.5-fold; $p = 0.874$) or after 21 d of exposure to EE2 at 1 ng/L (7-fold; $p = 1.00$) and 10 ng/L (28-fold; $p = 0.999$). Vitellogenin mRNA in female fish exposed to EE2 was not quantified.

2.3.4 Effects of MT/EE2 on kidney histopathology

KEH was higher in female and male fish exposed to 100 ng/L MT relative to control at 7 and 21 d (Fig. 2.4). A limited sample size (one to five fish/treatment) prevented a statistical

analysis from being conducted. However, an increase in KEH was consistently observed across the available samples of MT-exposed females (Fig. 2.4; Table 2.4). With the exception of altered KEH, no other histopathological effects of MT exposure were noted after 7 or 21 d in any treatment.

KEH was unchanged in female and male fish exposed to 100 ng/L EE2 (data not shown). KEH was not assessed at 1 and 10 ng/L of EE2 because no effect was observed at 100 ng/L. However, histopathological abnormalities were present in all fish exposed to 100 ng/L of EE2, regardless of sex (n = 6). Abnormalities included hyaline droplet accumulation and degeneration in the epithelium of the renal tubules, large eosinophilic deposits, eosinophilic deposits in the glomerular and renal tubules and necrosis of renal tubules (Fig. 2.5). None of these abnormalities were observed in either sex in the control group (n = 6).

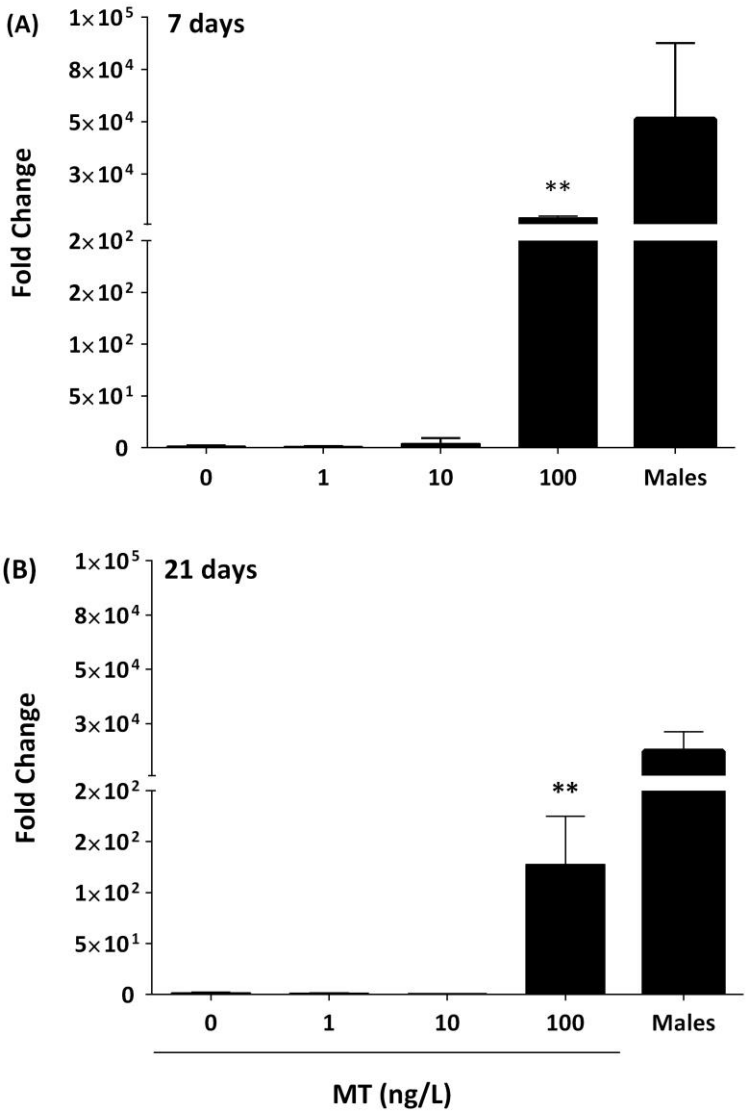

Fig. 2.2 Effect of 17α-methyltestosterone (MT) exposure on spiggin transcript abundance after (A) 7 days and (B) 21 days of exposure in the kidney of brook stickleback (*Culaea inconstans*). Females were exposed to MT (1, 10, 100 ng/L) for 7 or 21 days. Spiggin mRNA was measured in duplicate in experimental groups of 6-8 fish. Data were statistically analyzed with a one-way ANOVA followed by a post-hoc Dunnett's test and are shown as fold change (mean ± SEM) relative to the acetone-carrier control (0 ng/L) with significant differences indicated by an asterisk (** $p < 0.01$). Control male spiggin mRNA is shown for comparison but was not included in the statistical analysis.

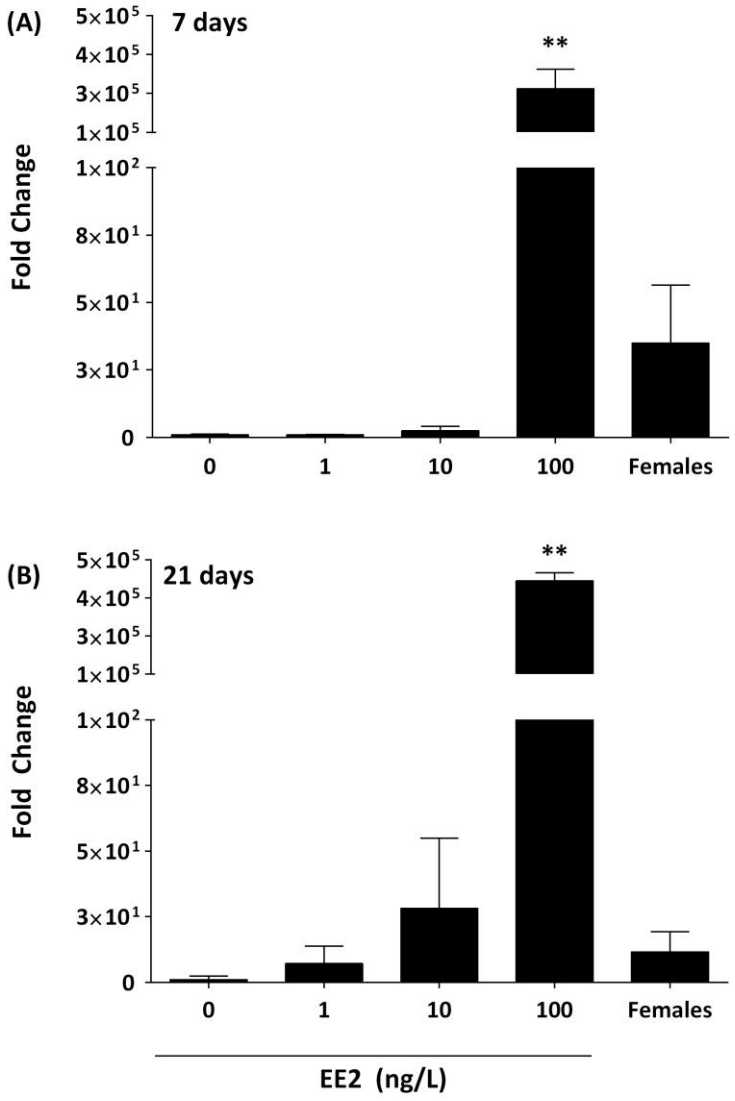

Fig. 2.3 Effect of 17α-ethinylestradiol (EE2) exposure on vitellogenin transcript abundance after (A) 7 days and (B) 21 days of exposure in the liver of brook stickleback (*Culaea inconstans*). Males were exposed to EE2 (1, 10, 100 ng/L) for 7 or 21 days. Vitellogenin mRNA was measured in duplicate in experimental groups of 4-8 fish. Data was statistically analyzed with a one-way ANOVA followed by a post-hoc Dunnett's test and are shown as fold change (mean ± SEM) relative to the acetone-carrier control (0 ng/L) with significant differences indicated by an asterisk (** $p < 0.01$). Control female vitellogenin mRNA is shown for comparison but was not included in the statistical analysis.

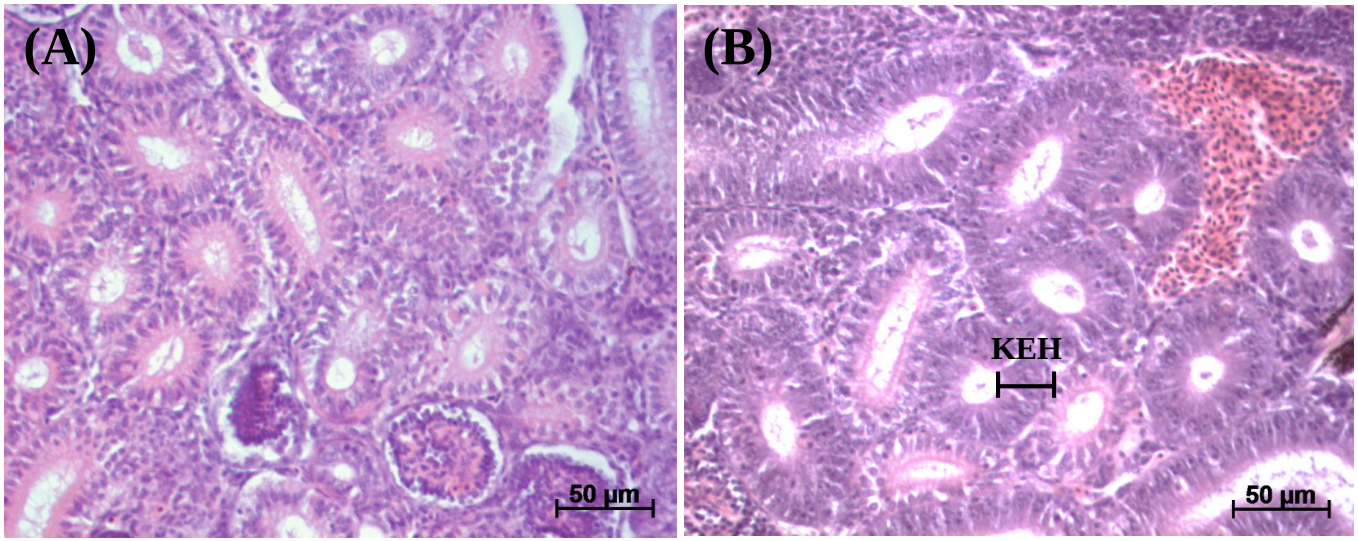

Fig. 2.4 Sections of female brook stickleback (*Culaea inconstans*) kidneys exposed to (A) 0 ng/L or (B) 100 ng/L of 17α-methyltestosterone (MT) for 7 days. Sections were cut at 5 µm thickness and stained with hematoxylin-eosin. The scale indicated in the bottom right corner is 50 µm. Kidney epithelium cell height increased from (A) 0 ng/L; KEH = 16.2 µm to (B) 100 ng/L; KEH = 26.3.

Table 2.4 Mean ± SE (n) of kidney epithelium cell height for female brook stickleback (*Culaea inconstans*) exposed to 17α-methyltestosterone for 7 and 21 days. Treatments are MT 1 ng/L (MT 1), 10 ng/L (MT 10) and 100 ng/L (MT 100).

Treatment Duration	Control	MT 1	MT 10	MT 100
7 days	16.2 ± 0.75 (4)	13.6 (1)	15.9 ± 1.24 (4)	26.3 ± 0.53 (2)
21 days	14.0 ± 0.35 (5)	21.1 ± 1.29 (2)	19.6 ± 1.30 (3)	21.7 ± 2.45 (3)

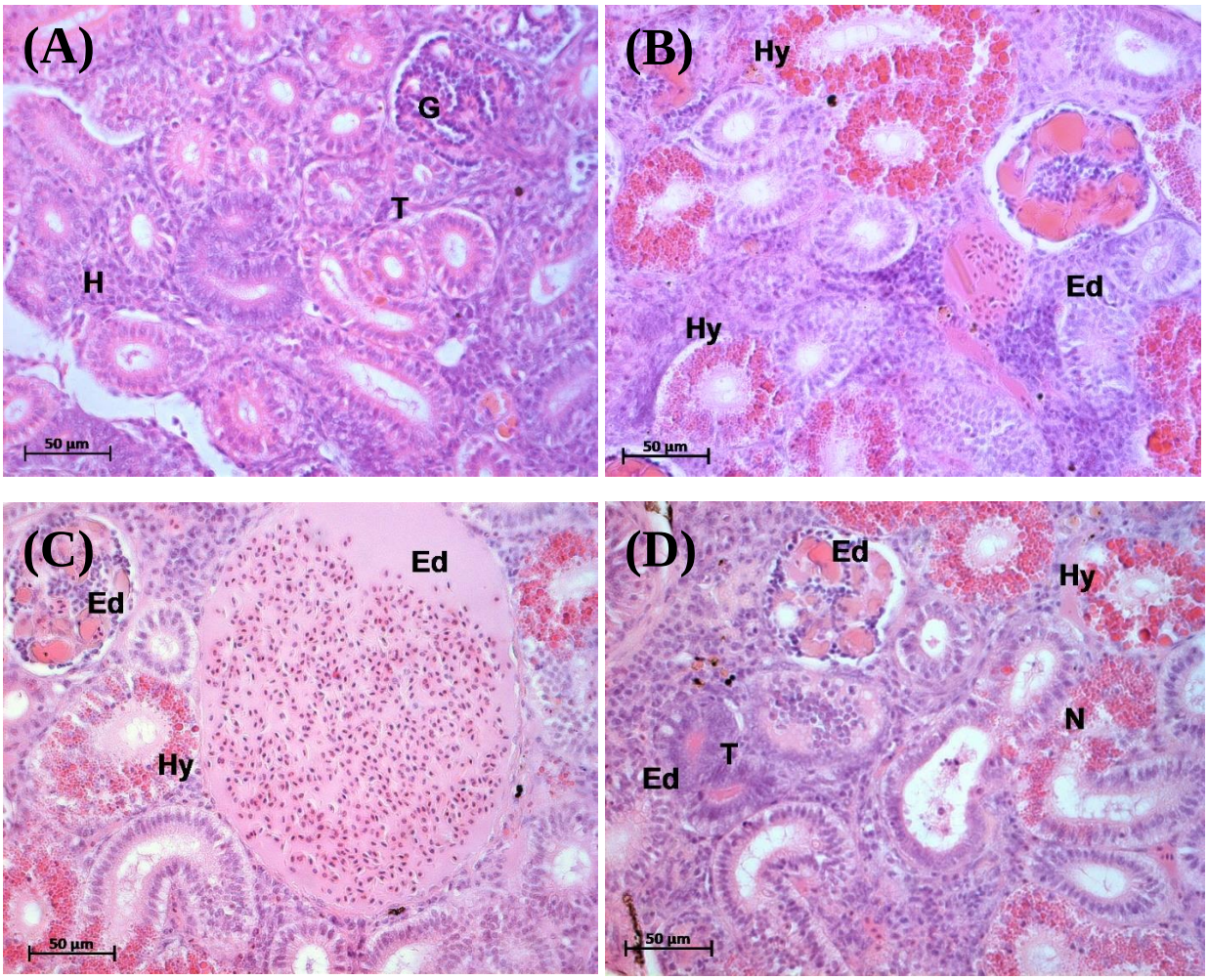

Fig. 2.5 Sections of brook stickleback (*Culaea inconstans*) kidneys (a) control glomerulus, distal and proximal tubules and interstitial hemopoietic tissue (b) stickleback exposed to 100 ng/L of 17α-ethinylestradiol (EE2) for 21 days with hyaline droplets in the tubules, eosinophilic deposits in the glomerulus and interstitial tissue (c) stickleback exposed to 100 ng/L of EE2 for 21 days with hyaline droplets in the tubules and large eosinophilic deposits and (d) stickleback exposed to 100 ng/L of EE2 for 21 days with tubule necrosis, eosinophilic deposits in the glomerulus and tubules and hyaline droplets. Sections stained with haematoxylin-eosin and cut at 5 µm thickness. Scale indicated in bottom left corner. Glomerulus (G); tubules (T), interstitial hemopoietic tissue (H); hyaline droplets (Hy); eosinophilic deposits (Ed), necrosis (N).

2.4 Discussion

This study is the first to evaluate molecular and apical changes in brook stickleback in response to hormone exposure. Vitellogenin and spiggin were differentially expressed in male and female brook stickleback. Exposure to MT resulted in significant increases in spiggin transcripts in females and exposure to EE2 resulted in significant increases in vitellogenin transcripts in males. An androgenic response was also observed through an increase in KEH in MT-exposed females. Organosomatic indices changed with EE2 exposure – HSI increased and GSI decreased in both sexes and in females, respectively. MT exposure did not affect organosomatic indices. Molecular and histological endpoints were the most sensitive responding after only 7 days of exposure whereas organosomatic indices either responded after 21 days (in the case of EE2 exposure) or not at all (in the case of MT exposure).

Sex-specific differences in the expression of spiggin and vitellogenin were clearly indicated in wild-caught male and female brook stickleback. Basal levels of spiggin mRNA in kidneys from mature brook stickleback were 17-fold higher in males relative to females. Although this demonstrates clear sex-specific expression, with spiggin transcript levels significantly higher in males than in females, the magnitude difference between sexes is much smaller compared to previous studies. In studies with threespine stickleback, Katsiadaki et al., (2006) and Hogan et al. (2008) reported levels of spiggin that were approximately four to five orders of magnitude higher in males compared to females. We also observed a sexually dimorphic expression pattern for vitellogenin in the liver, where expression was 72-fold higher in females than in males, but this difference was also small compared with previous studies in threespine stickleback (2-4 orders of magnitude; Geoghegan et al., 2008; Hogan et al., 2008). Sampling time of wild-caught fish was likely a major factor in the smaller difference observed in

basal transcript levels between sexes for brook stickleback. For example, Hogan et al. (2008) sampled threespine stickleback in the early breeding period in June whereas fish used in this study to characterize sex-differences in transcript levels were sampled in late August. Previous observations in threespine stickleback indicate that spiggin and vitellogenin levels begin to decline in June during the annual reproductive cycle of this species (Katsiadaki et al., 2006; Sokołowska and Kulczykowska, 2006). However, despite sampling later in the breeding season when basal expression of spiggin and vitellogenin was quite low, our results clearly show that expression of these transcripts in brook stickleback is sex specific.

Using the real-time PCR assay described and validated here, a dramatic induction of spiggin with exposure to 100 ng/L MT and vitellogenin with exposure to 100 ng/L EE2 is detectable in brook stickleback after 7 days. Notably, a few individual fish had increased transcripts in the 10 ng/L MT (7 days) and EE2 (7 and 21 days) exposure groups; however, inter-individual variability in response at 10 ng/L was such that there was no significant treatment effect. Given that MT and EE2 were not tested between 10 and 100 ng/L the lowest observable effect concentration (LOEC) for transcript induction may be lower than 100 ng/L. Nevertheless, with a clear, quantifiable androgenic response at 100 ng/L, spiggin transcript induction is among the most sensitive biomarkers for androgen exposure. Androgenicity of MT has often been identified using tubercle formation on exposed female fathead minnows but LOECs for this endpoint measured under flow-through exposure conditions were greater or equal to 1 μg/L (Hornung et al., 2004; Pawlowski et al., 2004a). In studies using the threespine stickleback, spiggin mRNA was induced with exposure to 10 ng/L of MT after 7 days (Hogan et al., 2008) while vitellogenin mRNA was induced with exposure to 10 ng/L of 17β-estradiol (E2) after 7 days (Hogan et al., 2008) and 18 ng/L of EE2 after 4 days (Katsiadaki et al., 2010). Although

species-specific differences may play a role in sensitivity to hormone exposure, differences in LOECs among studies could also be attributed to differences in reproductive status of fish and time of year that the exposure is conducted as well as exposure conditions (e.g. flow-through versus static-renewal). Brook stickleback in the present study were collected from the field in September and would have been in late gonadal recrudescence based on data from wild caught fish in the region (Tetreault et al., 2012). Gonadal recrudescence is characterized by rapid gonadal growth and maturation and is a stage that is sensitive to hormone-active compounds in other species (MacLatchy et al., 2003; Pawlowski et al., 2004a; Sharpe, 2004). Using fish sampled in June-July during early gonadal recrudescence may increase the sensitivity of these molecular endpoints to exogenous hormone exposure.

Both HSI and GSI changed in brook stickleback after 21 days of exposure to EE2. An increase in HSI at 100 ng/L of EE2 is consistent with reports in other species, including the threespine stickleback (Andersson et al., 2007), zebrafish (*Danio rerio*; Urbatzka et al., 2012; Van den Belt et al., 2002) and medaka (*Oryzias latipes*; Ma et al., 2007). The liver plays an active role in vitellogenin synthesis and exposure to EE2 has been previously associated with an increased HSI due to vitellogenesis induction (Andersson et al., 2007). A reduction in GSI was observed with exposure to all EE2 treatments in female brook stickleback after 21 days. Studies using threespine stickleback (Andersson et al., 2007), zebrafish (Van den Belt et al., 2002) and fathead minnow (Filby et al., 2007), in comparable exposure scenarios, have demonstrated similar effects on GSI with exposure to EE2. Reduced GSI in EE2-exposed female fish has been attributed to arrested ovarian development and degeneration (Pawlowski et al., 2004b; Van den Belt et al., 2002). Estrogenic chemicals can act through a negative feedback pathway to reduce the release of gonadotropin-hormone II from the pituitary which could arrest ovarian

development leading to follicular atresia and ovarian degeneration (Van Der Kraak et al., 1998). Alternatively, they may act directly on the ovary to disrupt sex steroid production which could similarly arrest ovarian development and lead to cellular degeneration (Van Der Kraak et al., 1998). The mechanism for reduced GSI in female brook stickleback exposed to EE2 cannot be established without supporting gonad histology. Therefore, future studies could incorporate gonad histology as an endpoint in establishing the response of brook stickleback to estrogenic chemical exposure. Overall, a change in organosomatic indices and the supporting induction of vitellogenin demonstrate an estrogenic response in EE2-exposed brook stickleback. These effects have been linked to reproductive impairment in an EE2-exposed fathead minnow population (Kidd et al., 2007) and could result in similar outcomes in brook stickleback; however, this remains to be studied.

Increased KEH and NSI are androgen-specific responses in threespine stickleback that have been used to determine the androgenicity of compounds (Borg et al., 1993; Katsiadaki et al., 2002a, 2002b; Sanchez et al., 2008a). In brook stickleback, androgen exposure resulted in kidney hypertrophy as indicated by increased KEH in female brook stickleback exposed to 100 ng/L of MT for 7 days. Kidney hypertrophy in male stickleback occurs because of an increase in epithelium cell height of the secondary proximal convoluted tubules (site of spiggin production) in response to increasing concentrations of circulating androgens (Jakobsson et al., 1999). Borg et al. (1993) was the first to show that KEH in castrated male threespine stickleback increased with exposure to several androgenic compounds. Subsequent research demonstrated that KEH and spiggin production are positively correlated and can be induced in female threespine stickleback exposed to exogenous androgens (Katsiadaki et al., 2002a; Wartman et al., 2009). Likewise, our results demonstrate that KEH and spiggin production in brook stickleback are

responsive indicators of androgen exposure. Surprisingly, MT exposure did not affect NSI which has been shown to increase with exposure to 100 ng/L of MT in threespine stickleback (Sanchez et al., 2008a). NSI may not be as sensitive an indicator of androgen exposure as histopathological effects. In addition, the ability of an androgenic compound to alter NSI may depend on the reproductive status of male and female stickleback. NSI in male stickleback varies seasonally with reproductive status (Sokołowska and Kulczykowska, 2006) and, although the reproductive status of stickleback can be manipulated with a lengthened photoperiod, photosensitivity can be affected by season whereby some fish do not mature under a lengthened photoperiod due to strong circannual rhythms (Baggerman, 1985). The size of kidneys and gonads in the MT-exposed males and females were small indicating that they were likely in an early reproductive state (Sokołowska et al., 2004) and this may have made it more difficult to discern the effects of MT on NSI. Although NSI did not respond to MT exposure, the morphological data of increased KEH coupled with increased spiggin mRNA levels is evidence that exposure to an exogenous androgen elicits a clear, functional androgenic response in brook stickleback.

Histopathological assessment of kidneys in EE2-exposed fish was not an original objective of this study – however, upon examination we noticed abnormalities in kidneys sampled from male and female stickleback exposed to 100 ng/L of EE2. Similar nephrological abnormalities have been observed in other fish species exposed to estrogenic compounds, such as fathead minnow (Länge et al., 2001; Palace et al., 2002), zebrafish (Weber et al., 2003) and rainbow trout (Herman and Kincaid, 1988). Nephrological abnormalities in estrogen-exposed fish have been attributed to vitellogenin production. For example, Wester et al. (1985) found that hyaline droplet formation and eosinophilic depositions in kidney resemble egg-yolk. Based on this finding, others have postulated that hyaline droplets form following resorption of large

proteins (such as vitellogenin) in the epithelium cells of the renal tubules (Oropesa et al., 2013; Palace et al., 2002; Weber et al., 2003; Zaroogian et al., 2001). Additionally, large eosinophilic droplets may be a result of vitellogenin leakage from overwhelmed renal tubules which are unable to filter the large volumes of plasma vitellogenin produced during exposure to exogenous estrogens (Herman and Kincaid, 1988; Zaroogian et al., 2001). Tubule necrosis and degeneration have also been noted with exposure to EE2 and E2 (Herman and Kincaid, 1988; Oropesa et al., 2013; Weber et al., 2003). Consequently, the histopathological effects in the kidney of fish in the present study are likely a secondary effect of increased vitellogenin production, which is supported by the high vitellogenin transcript levels measured in EE2-exposed fish.

The results of this study suggest that spiggin in brook stickleback is a sensitive and quantifiable biomarker for androgen exposure compared to androgenic biomarkers in other small model fish. However, comparison of spiggin induction in brook stickleback to androgenic responses in other small fish models exposed to MT should be made with caution due to differences in the exposure conditions between this study (semi-static renewal) and other studies (flow-through). In fathead minnow, exposed under flow-through ≥ 1 µg/L MT was required to induce significant tubercle formation in females (Ankley et al., 2001; Hornung et al., 2004; Pawlowski et al., 2004a). In Japanese medaka, exposed under flow-through, the formation of papillary processes (a male-specific secondary sexual characteristic) in females occurred at 46.8 ng/L of MT (Kang et al., 2008). Although papillary formation in medaka is a more sensitive endpoint than spiggin in brook stickleback (based on the results herein), MT concentrations between 10 and 100 ng/L were not tested in this study and so the LOEC for spiggin induction in brook stickleback may fall below 100 ng/L. Also, spiggin reduction in androgenized female threespine stickleback is a sensitive indicator of anti-androgen exposure whereas the reduction of

papillary processes in male medaka is not (Katsiadaki et al., 2006; Nakamura et al., 2014; OECD, 2006). Furthermore, spiggin induction is androgen-dependent unlike gonadal structure and fecundity, which have also been shown to be affected by MT exposure at concentrations ≥ 0.1 µg/L (Kang et al., 2008; Pawlowski et al., 2004a). In the case of the fathead minnow, studies no longer use MT as a model androgen because the concentrations required to induce tubercle formation also result in estrogenic effects (such as vitellogenin induction; Ankley et al., 2001; Hornung et al., 2004; Pawlowski et al., 2004a). This is likely due to the ability of MT to be aromatized to methylestradiol, resulting in estrogenicity (Ankley et al., 2001; Hornung et al., 2004). The concentrations of MT employed in the present study induced spiggin but not vitellogenin. Likewise, MT had no effect on vitellogenin mRNA in threespine stickleback under similar exposure conditions (Hogan et al., 2008). It appears that in stickleback, MT is not aromatized into methylestradiol at concentrations high enough to induce vitellogenin in males. Future studies should explore the relative potency of other androgenic compounds (e.g., 5α-dihydrotestosterone, 11-ketotestosterone). Additional research is also required to confirm the sensitivity of brook stickleback to MT and EE2 using comparable exposure scenarios (ie. flow-through) in order to further validate spiggin as an androgenic biomarker in this species. In terms of estrogen responsiveness, the fathead minnow, medaka and zebrafish may be more suitable test species with more established test methods and (in some cases) a more sensitive vitellogenin response than stickleback (reviewed in Katsiadaki et al., 2007). However, the ability to simultaneously assess androgen and estrogen exposure in a single fish using fully quantitative endpoints is an advantage held only by the stickleback.

The suitability of brook stickleback as a bioindicator of EDCs is further enhanced by their broad distribution and physiology. Brook stickleback are abundant throughout North

America and are found in a range of freshwater environments (including lakes, streams and ponds; (Stewart et al., 2007). They are fairly sedentary and are therefore good indicators of environmental conditions within a given location (Wootton, 1984). They can survive low oxygen, cold water temperatures and repeated handling (Reisman and Cade, 1967; Wootton, 1984) and this tolerance may allow them to survive contaminated environments when other fish do not. They can also be bred in the laboratory (McKenzie, 1969; McLennan, 1993; Reisman and Cade, 1967) and so could be used in reproductive bioassays that evaluate effects of EDC exposure on reproductive behaviour and fecundity. The definitive, measurable responses to hormone exposure demonstrated here extend the use of brook stickleback to regulatory testing and ecological monitoring of endocrine active compounds in aquatic environments. Furthermore, there is potential to develop additional endocrine bioassays for brook stickleback, similar to those used with threespine and other species, such as in vitro gonadal steroidogenesis (Hogan et al., 2008; MacLatchy et al., 2003), gonad histopathology (Allen et al., 2008), behaviour (in stickleback related to male nest building and courting; Dzieweczynski and Forrette, 2014; Sebire et al., 2008) and fecundity (Maunder et al., 2007). Reproductive and behavioural endpoints in brook stickleback could then be utilized in constructing adverse outcome pathways, linking molecular indices to adverse outcomes that are relevant to risk assessment. There is also significant potential to assess the anti-androgenic properties of compounds using in vitro (kidney explants) and in vivo methods (21-day androgenised female stickleback assay) already established in the threespine stickleback (Jolly et al., 2009; Katsiadaki et al., 2006; OECD, 2011). Finally, both biomarker and apical endpoints could be evaluated in brook stickleback with exposure to environmental samples in both the field and laboratory. The threespine stickleback has been successfully employed as a biomonitoring species in contaminated environments and to

assess the endocrine activity of complex mixtures, including sewage and pulp and paper mill effluent (Björkblom et al., 2013, 2009; Katsiadaki et al., 2012; Pottinger et al., 2011; Sanchez et al., 2008b; Wartman et al., 2009).

2.5 Conclusion

In conclusion, we have developed two sensitive qPCR assays that can be used to measure either androgen exposure through spiggin transcript induction in female kidneys or estrogen exposure through vitellogenin transcript induction in male livers. We have demonstrated that these endpoints are highly responsive to short term hormone exposure. Moreover, androgenic and estrogenic responses of brook stickleback were measurable at higher levels of biological organization (e.g. organosomatic indices). As such, we were able to demonstrate a clear, definitive androgenic and estrogenic response, which was detected not only at the molecular level but also through morphological endpoints that corroborate an androgenic and estrogenic response. The ability to evaluate whole organism effects and mechanisms of action positions the brook stickleback as a promising new freshwater fish model for understanding (anti)androgenic responses in fish.

Acknowledgements

We thank Dr. Jason Raine and the Aquatic Toxicology Research Facility (ARTF) at the Toxicology Centre, University of Saskatchewan for providing space and equipment for the exposure study. We also thank Drs. Steve Wiseman and Tim Jardine for comments on the manuscript and various members of the Hogan lab for assistance with field and lab work. This research was funded by a Natural Sciences and Engineering Research Council of Canada

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CHAPTER 3

3. EVALUATION OF THE ANTI-ANDROGENIC EFFECTS OF FLUTAMIDE USING AN ANDROGENIZED FEMALE BROOK STICKLEBACK (CULAEA INCONSTANS) BIOASSAY[2]

[2]This chapter will be submitted for publication to Aquatic Toxicology under co-authorship with Dr. Natacha Hogan (University of Saskatchewan). B. Muldoon designed and conducted the experiments, collected and analyzed the data and wrote the manuscript. N. Hogan supervised B. Muldoon and provided support throughout the exposure, and assisted in the preparation of the manuscript. The tables, figures and references cited herein have been reformatted to adhere to the thesis style. References for this chapter can be found in the reference section for this thesis.

3.1 Introduction

Chemicals that enter the aquatic environment pose a risk to aquatic organisms through their interaction and interference with the endocrine system. These chemicals, known as endocrine disrupting compounds (EDCs), cause adverse effects in both laboratory and wild fish including impaired reproduction, behaviour, and development (reviewed in Arukwe, 2001; Mills and Chichester, 2005; Sumpter, 2002). Initial investigations into EDCs focussed on estrogenic chemicals but has since expanded to include (anti-)estrogenic and (anti-)androgenic chemicals. Masculinization of female mosquito fish found downstream of a pulp and paper mill operation was the first recorded incident of endocrine disruption in wild fish attributed to androgenic chemicals (Howell et al., 1980). Recently, there has been increasing concern that agonists and antagonists of the androgen receptor (AR) can enter aquatic receiving environments via sources such as municipal effluent treatment facilities (Wen et al., 2013) and agricultural runoff (Kolok and Sellin, 2008). The feminization of wild fish populations was previously attributed to exposure to estrogenic chemicals from municipal wastewater (Jobling et al., 2009). However, substantial anti-androgenic activity has since been detected in treated municipal wastewater and suggested to be a contributing factor in the feminization of wild fish (Jobling et al., 2009). Therefore, biomarkers of exposure to anti-androgenic chemicals need to be identified in fish to thoroughly evaluate the risk EDCs pose to aquatic organisms.

Several chemicals, including pesticides, pharmaceuticals and personal care products, and industrial pollutants, have been identified as having anti-androgenic activity (reviewed in Chambers et al., 2002.; Environment Canada, 2011; Johnson et al., 2007; Kleywegt et al., 2007). A central mechanism of action for anti-androgenic chemicals is through androgen-receptor antagonism, whereby chemicals bind to and block the androgen receptor preventing activation by

endogenous androgens (Scholz and Mayer, 2008). Anti-androgenic effects in fish have been demonstrated in several laboratory studies. For example, exposure to flutamide (a model androgen-receptor antagonist) resulted in demasculinized male guppies (*Poecilia reticulate*; Baatrup and Junge, 2001) and reduced nesting and courting behaviour in male threespine stickleback (*Gasterosteus aculeatus*; Sebire et al., 2008). Other anti-androgenic responses in fish include reduced body size, skewed sex ratios towards females, decreased fecundity, altered secondary sexual characteristics and sexual behaviour, reduced sperm counts, reduced gonad size (relative to fish weight; GSI) and smaller oocytes (reviewed in Mills and Chichester, 2005). However, it is difficult to link an adverse effect in fish (especially in wild-fish) to exposure to exogenous anti-androgens (Sumpter and Johnson, 2008). Developing (anti-)androgen-specific biomarkers in fish may aid in screening for anti-androgenic activity of chemicals and determining their potential risk to fish reproduction.

Spiggin in stickleback is among the few biomarkers used in the assessment of androgenic chemicals (reviewed in Katsiadaki et al., 2007). Spiggin is a glycoprotein used in nest-building and produced in the kidney of male stickleback during the reproductive season when endogenous androgen levels are high (Jakobsson et al., 1999). Spiggin production is accompanied by structural changes to the kidney and initiation of courting and nesting behaviour (Sokołowska and Kulczykowska, 2006). Exposure to exogenous androgenic chemicals can also induce spiggin production in the kidney of female threespine (Katsiadaki et al., 2002a) and brook stickleback (*Culaea inconstans*; Chapter 2). Conversely, exposure to anti-androgenic compounds reduces spiggin production in androgenized female stickleback (Hogan et al., 2012; Katsiadaki et al., 2006). This method is considered a robust approach in demonstrating the anti-androgenic potency of chemicals (Katsiadaki and Sebire, 2011) and is the basis for the Androgenized

Female Stickleback Screen (AFSS; OECD, 2011). In the AFSS females are exposed to an androgen to induce spiggin production and simultaneously exposed to a range of concentrations of a putative anti-androgen to suppress spiggin production (OECD, 2011). Anti-androgenic potency is then determined based on the concentration required to significantly reduce spiggin production (OECD, 2011). High inter-individual variability in male spiggin expression outside of spawning has led to the use of females in the anti-androgen assay (Katsiadaki et al., 2006).

In North America, the fathead minnow (*Pimephales promelas*) is the commonly used small fish model to assess EDCs. The appearance of nuptial tubercles in female fathead minnow (a secondary sexual characteristic only observed in males) is used to indicate androgen exposure whereas a tubercle reduction in males is used to indicate anti-androgen exposure (Jensen et al., 2004; Martinović et al., 2008; Panter et al., 2004). However, reduced nuptial tubercles in males exposed to an anti-androgenic chemical is not consistently observed across studies (Jensen et al., 2004; Martinović et al., 2008; Panter et al., 2004). A reduction in nuptial tubercles in masculinized female fathead minnow is another method used to assess anti-androgenic chemicals (Ankley et al., 2004). However, this method has a small dynamic range and a low sensitivity through which anti-androgenic potency can be quantified (Katsiadaki and Sebire, 2011). In comparison, spiggin is a sensitive and highly responsive biomarker used to detect exposure to chemicals with (anti-)androgenic activity. Reduced spiggin in androgenized females is consistently observed with exposure to synthetic and environmentally-relevant anti-androgenic compounds (reviewed in Katsiadaki et al., 2007) and a 1000-fold change in spiggin in exposed female stickleback provides a wide-range under which anti-androgen potency can be assessed (Katsiadaki and Sebire, 2011). Within North America this assay is not typically used for biomonitoring of (anti-)androgenic EDCs likely due to the fact that the habitat of the threespine

stickleback is limited to estuarine and coastal waters. Obtaining this species for testing may be difficult in other parts of North America.

Alternatively, brook stickleback are a small, abundant freshwater fish in North America whose males produce spiggin which can be induced in females exposed to an exogenous androgenic chemical (Chapter 2). The anti-androgenic response of brook stickleback has not been characterized. Therefore, the objective of this study was to assess the responsiveness of androgenized brook stickleback to chemical exposure to an anti-androgen. A short-term co-exposure to 17α-methyltestosterone (MT) and flutamide (FL) was conducted to determine the concentration of FL required to antagonize the androgenic response to MT under semi-static renewal conditions. Molecular (spiggin mRNA) and apical biomarkers (organosomatic indices, condition factor and kidney histology) were measured to characterize their responses. MT was chosen as the model androgen because its action can be antagonized with exposure to an anti-androgen and its concentration remains stable for 48 hours in a waterborne exposure (Katsiadaki et al., 2006). FL was chosen as the anti-androgen because it has an established mechanism of action through androgen receptor antagonism and is an effective anti-androgen in fathead minnow (Ankley et al., 2004; Jensen et al., 2004) and threespine stickleback (Katsiadaki et al., 2006). Sampling was conducted after 4 and 14 days of exposure to determine if responsiveness would vary with length of exposure.

3.2 Material and Methods

3.2.1 Animals

Adult brook stickleback were caught with dip nets between June and August 2014 from Chappell Marsh Conservation Area, Saskatchewan. Fish were transported to the Aquatic

Toxicology Research Facility (Toxicology Centre, University of Saskatchewan, Canada) and placed in a flow-through 84" x 24" x 14.5" Min-O-Cool with filtered facility water. Fish were maintained at 16 ± 1 °C and held at a 12:12 (light:dark) photoperiod until late July at which point the photoperiod was gradually adjusted to 16:8 (light:dark) in order to stimulate spawning conditions. Fish were fed frozen bloodworms twice daily (Sally's Bloodworms, Sanfrancisco Bay Brand, CA, United States). The University of Saskatchewan's Animal Research Ethics Board approved all methods used in this study (AUP #: 20130105) and the methods adhered to the Canadian Council on Animal Care guidelines for humane animal use.

3.2.2 Laboratory exposures and sampling

Stock chemicals, FL (CAS #: F9397) and MT (CAS #: 58-18-4), used for the exposure were purchased from Sigma-Aldrich (Oakville, ON, Canada). One week prior to exposure, fish were randomly selected and transferred from husbandry tanks to 37 L glass exposure tanks filled with 25 L of water. Each tank contained 25 fish, a loading density outlined in the OECD AFSS protocol (2011). Male and female fish were included in the exposure since it is difficult to externally distinguish sexes. Brook stickleback were co-exposed to MT and FL for 14 days. MT was administered at 500 ng/L, a dose that induces an androgenic response but can be antagonized with exposure to FL (Katsiadaki et al., 2006). FL was then administered from separate stock solutions at 25, 150 and 250 µg/L. Three controls were included: a positive control (500 ng/L MT), a negative control (250 µg/L FL) and an acetone-carrier control (0.002%). Exposures were semi-static with a 48-hour water renewal and re-dose. All treatments were conducted in triplicate. Water temperature was maintained at 16 ± 1 °C with a 16:8 (light:dark) photoperiod.

Fish were sub-sampled on day 4 (37 fish/treatment) and the remaining fish were sampled on day 14. Fish were stunned by a blow to the head, weighed and total length measured prior to

being euthanized by spinal severance. Gonads, kidneys and livers were excised, weighed (to the nearest 0.01 g), immediately flash-frozen on dry ice and stored at -80 °C until RNA extraction. A small subsample of fish was also collected from one tank from each treatment for kidney histology at day 14. Fish randomly assigned for histology were placed whole into 10% neutral-buffered formalin (a small abdominal incision was made to ensure infiltration of the preservative) and later transferred to 70% ethanol for long-term storage prior to processing.

3.2.3 RNA extraction and real-time PCR

RNA was obtained from the kidneys using the E.Z.N.A. MicroElute Total RNA Kit as described by the manufacturer (Omega BioTek, Norcross, GA, United States). Extracted RNA was resuspended in 20 µL of nuclease-free water and stored at -80 °C. To remove genomic DNA contamination, RNA was treated with Turbo DNA-free kit (Ambion, Burlington, ON, Canada). RNA quantification was determined on a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, United States). To confirm RNA quality, total RNA was run on a 1% tris-borate-EDTA agarose gel and visualized with ethidium bromide. Reverse transcription was run using iScript cDNA synthesis kit with an RNA input of 0.25 µg for the kidneys according to the manufacturer (Bio-Rad Laboratories Inc., Hercules, CA, United States). The cDNA reaction was diluted 25-fold and stored at -20 °C prior to use in gene expression analysis.

Real-time quantitative polymerase chain reaction (qPCR) was conducted using gene-specific primers for spiggin and two reference genes, 18S and ribosomal-protein L8 (RPL-8), using the protocol described in Chapter 2. Briefly, real-time qPCR was run using a CFX96 Real-time C1000 Thermal Cycler (Bio-Rad) with 20 µL master mix reactions containing the following: 1x SsoFast EvaGreen Supermix (Bio-Rad), 0.4 mM each of forward and reverse primers (Invitrogen), and 2 µL of diluted cDNA template. The thermocycling program for qPCR

was run as: initial denaturation for 30 s at 95 °C followed by 40 cycles of 5 s at 95 °C and 20 s at gene-specific annealing temperature (RPL-8 and spiggin: 60 °C; 18S: 62 °C); 1 min at 95 °C; and then melt curve analysis starting at 55 °C with a 1 °C incremental increase every 5 s to 95 °C. Standard curves, using serially-diluted cDNA, were run on every plate for each primer and accepted with efficiencies of 90-110% and $R^2 \geq 0.98$. The relative standard curve quantification method (Applied Biosystems, 1997) was used to determine the relative abundance of spiggin mRNA in the kidney (all samples run in duplicate). No-reverse transcriptase and no-template controls were included on every plate. Abundance of spiggin mRNA was normalized against 18S and RPL-8 and the geometric mean was used to calculate fold-change per treatment. Data are expressed as mean fold-change (relative to acetone-carrier control) ± standard error.

3.2.4 Morphometrics and kidney histology

Condition factor (CF) was calculated using Fulton's *K* (Ricker, 1975). Liver, gonad and kidney weights were used to calculate hepatosomatic index (HSI), gonadosomatic index (GSI) and nephrosomatic index (NSI) (respectively) using the following formula: (organ weight/fish weight) x 100. Kidney epithelium cell height (KEH) was measured from histological samples of fish kidneys collected at 14 days of exposure. The methodology was carried out as described in Chapter 2.

3.2.5 Statistical analysis

Data were tested for normality using Kolmogorov-Smirnov test on the residuals and for homogeneity of variance using Levene's test. Data not meeting the assumptions were log-transformed and re-tested. Morphometric data (OSI and CF) were analyzed separately for females and males using a nested ANOVA whereby tank was nested in treatment. If tank-factor

p-value was ≥ 0.250 then tank was removed as a factor and the data were re-run as a one-way ANOVA. Hormone-induced gene expression data (mRNA abundance) were analyzed using a one-way ANOVA. Post-hoc analysis on all data was conducted with a multi-comparison post-hoc Tukey test. Statistical analysis was run on IBM SPSS Statistics 22 (Armonk, NY, United States). Differences among treatments were considered significant at $p \leq 0.05$.

3.3 Results

3.3.1 Mortality, length, weight and morphometrics

Mortality did not exceed 10% in any treatment (data not shown). There were no significant differences among treatments for length, weight, CF and organosomatic indices in males or females after 4 d of exposure (Table 3.1; Fig.3.1A, 3.2A, 3.3A, 3.4A). Similarly, CF, length, weight and GSI did not significantly differ among treatments after 14 d of exposure (Table 3.1). However, after 14 d there was a reduction in HSI in co-treated females which was significant at all concentrations relative to the solvent-control and in select concentrations relative to MT- and FL-controls ($p = 0.049$; Fig. 3.1B). Similarly, HSI in males was significantly reduced in select co-treatments relative to the solvent-control and in all co-treatments relative to FL-control ($p = 0.007$; Fig. 3.2B). HSI in males did not significantly differ among co-treatments or among positive and negative controls. Likewise, HSI did not differ significantly between co-treated males and the MT-only control (Fig. 3.2B).

NSI in females was significantly higher in MT-control and co-treated fish relative to the solvent- and FL-controls after 14 d ($p = 0.001$; Fig. 3.3B). Also in females, as FL concentrations increased in the co-treated fish, there was a trend towards lower NSI but these differences were not significant (Fig. 3.3B), and co-treated females were also not different from the MT-control.

Similarly in males, NSI was significantly higher in MT-control and co-treated fish relative to solvent- and FL controls after 14 d ($p = 0.001$; Fig. 3.4B). NSI was significantly higher in the 25 µg/L FL co-treated males relative to the MT-control. However, there were no significant differences between the 150 and 250 FL co-treatments and the MT-control. As FL concentrations increased in co-treated males there was a significant, dose-dependent reduction in NSI that was significant between co-treatments 25 and 250 µg/L FL (Fig. 3.4B).

3.3.2 Spiggin transcript levels

After 4 and 14 d of exposure, spiggin transcript levels in female kidneys were significantly increased by four orders of magnitude in the MT-control relative to solvent-control ($p = 0.001$; Fig. 3.5A&B). Levels of spiggin mRNA did not significantly differ between the solvent-control and FL-control (Fig. 3.5A&B). There was no significant difference in spiggin transcript levels between co-treatments and the MT-control after either 4 or 14 d. However, after 4 d a slight, but insignificant, decline in spiggin transcript levels occurred between co-treated females; 25 to 150 µg/L FL (20 % decrease) and 25 to 250 µg/L FL (60 % decrease; Fig. 3.5A). Similarly, after 14 d of exposure a slight but insignificant decrease in spiggin transcript levels was detected between co-treated females; 25 to 150 µg/L FL (52 % decrease) and 25 to 250 µg/L FL (60 % decrease; Fig. 3.5B). Therefore, the concentrations of FL administered to co-treated female fish were not sufficient to ameliorate MT-induced spiggin expression after either 4 or 14 d. Spiggin mRNA was not measured in exposed male fish at either 4 or 14 d.

Table 3.1 Mean (± SE) length, weight, condition factor (CF) and gonadosomatic index (GSI) in female and male brook stickleback (*Culaea inconstans*) exposed to 17α-methyltestosterone and flutamide for 4 and 14 days. Treatments are solvent-control (control), 500 ng/L MT positive control (MT-CTRL), 250 µg/L positive control (FL-CTRL) and co-treatments to 500 ng/L MT and 25 µg/L FL (FL-25), 150 µg/L FL (FL-150) or 250 µg/L FL (FL-250).

Sex (treatment duration)	Variable	Control	MT-CTRL	FL-CTRL	FL-25	FL-150	FL-250
Female (4 day)	**Length (mm)**	4.2 ± 0.2	4.2 ± 0.2	4.5 ± 0.1	4.5 ± 0.2	4.4 ± 0.2	4.3 ± 0.1
	Weight (g)	0.59 ± 0.06	0.59 ± 0.07	0.67 ± 0.05	0.67 ± 0.07	0.64 ± 0.06	0.62 ± 0.06
	CF	0.70 ± 0.03	0.75 ± 0.02	0.72 ± 0.02	0.73 ± 0.01	0.74 ± 0.02	0.75 ± 0.01
	GSI (%)	1.4 ± 0.1	1.4 ± 0.2	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
Female (14 day)	**Length (mm)**	4.5 ± 0.2	4.1 ± 0.1	4.1 ± 0.1	4.2 ± 0.1	4.4 ± 0.1	4.3 ± 0.1
	Weight (g)	0.66 ± 0.03	0.52 ± 0.03	0.48 ± 0.03	0.55 ± 0.08	0.62 ± 0.05	0.61 ± 0.05
	CF	0.69 ± 0.02	0.75 ± 0.02	0.70 ± 0.02	0.72 ± 0.01	0.72 ± 0.01	0.75 ± 0.02
	GSI (%)	1.6 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	1.3 ± 0.1
Male (4 day)	**Length (mm)**	4.5 ± 0.1	4.6 ± 0.1	4.4 ± 0.1	4.6 ± 0.1	4.4 ± 0.1	4.5 ± 0.1
	Weight (g)	0.75 ± 0.07	0.79 ± 0.05	0.70 ± 0.06	0.73 ± 0.05	0.67 ± 0.05	0.70 ± 0.05
	CF	0.78 ± 0.03	0.78 ± 0.02	0.78 ± 0.03	0.75 ± 0.03	0.78 ± 0.02	0.75 ± 0.03
	GSI (%)	0.68 ± 0.11	0.69 ± 0.06	0.68 ± 0.05	0.68 ± 0.06	0.55 ± 0.05	0.73 ± 0.09
Male (14 day)	**Length (mm)**	4.3 ± 0.1	4.6 ± 0.1	4.4 ± 0.1	4.2 ± 0.2	4.3 ± 0.1	4.5 ± 0.2
	Weight (g)	0.62 ± 0.04	0.70 ± 0.05	0.65 ± 0.06	0.67 ± 0.07	0.64 ± 0.06	0.71 ± 0.07
	CF	0.76 ± 0.02	0.73 ± 0.02	0.72 ± 0.01	0.76 ± 0.01	0.76 ± 0.02	0.76 ± 0.01
	GSI (%)	0.72 ± 0.09	0.87 ± 0.09	0.68 ± 0.07	0.68 ± 0.09	0.71 ± 0.08	0.68 ± 0.08

Number of observations varied between 11 and 25 fish per group
No significant findings (α = 0.05)

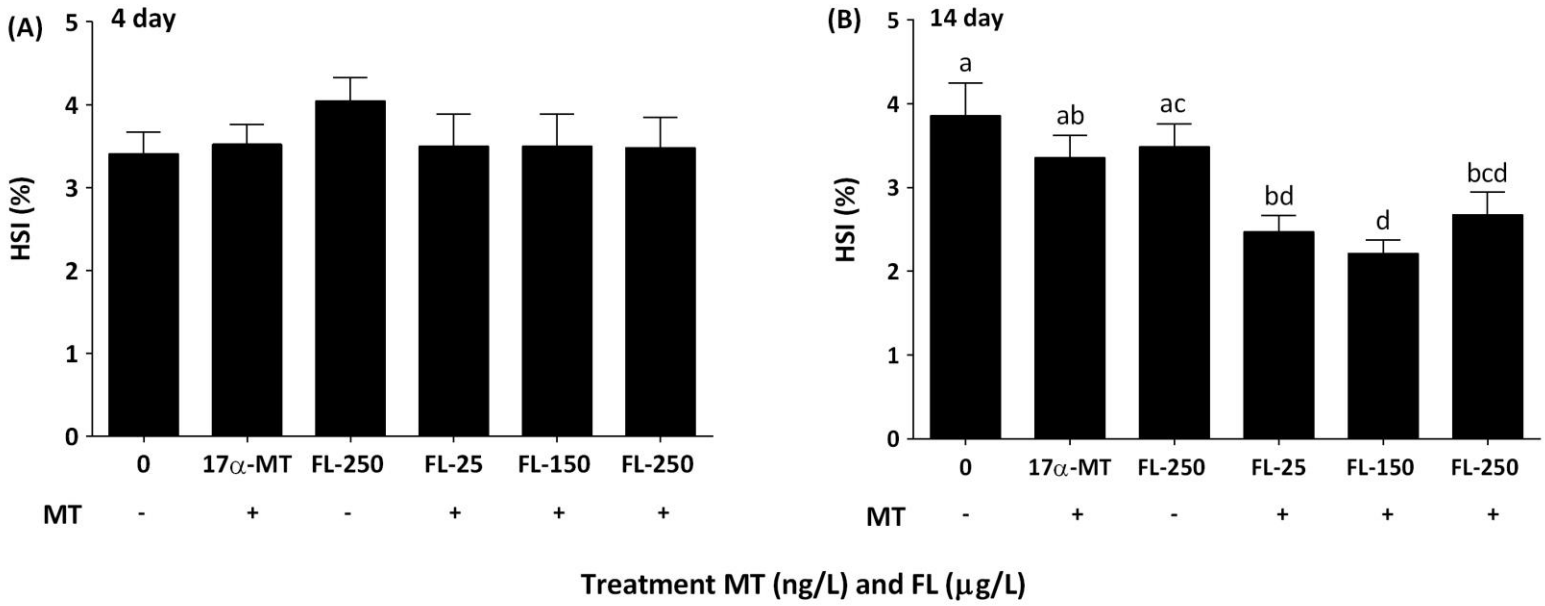

Fig. 3.1 Change in hepatosomatic index (HSI; mean ± SE) in female brook stickleback (*Culaea inconstans*) after (A) 4 days and (B) 14 days of exposure to an acetone-carrier control (0 ng/L), 17a-methyltestosterone (MT: 500 ng/L), flutamide (FL: 250 µg/L) or a combination of MT at 500 ng/L and FL at either 25, 150 and 250 µg/L. Morphometric data was analyzed using a two-way ANOVA with tank nested in treatment and re-run as a one-way ANOVA if the tank effect was not significant ($p \geq 0.250$). Post-hoc analysis was run as a Tukey test. Treatments shown with different letters are significantly different from one another ($p < 0.05$).

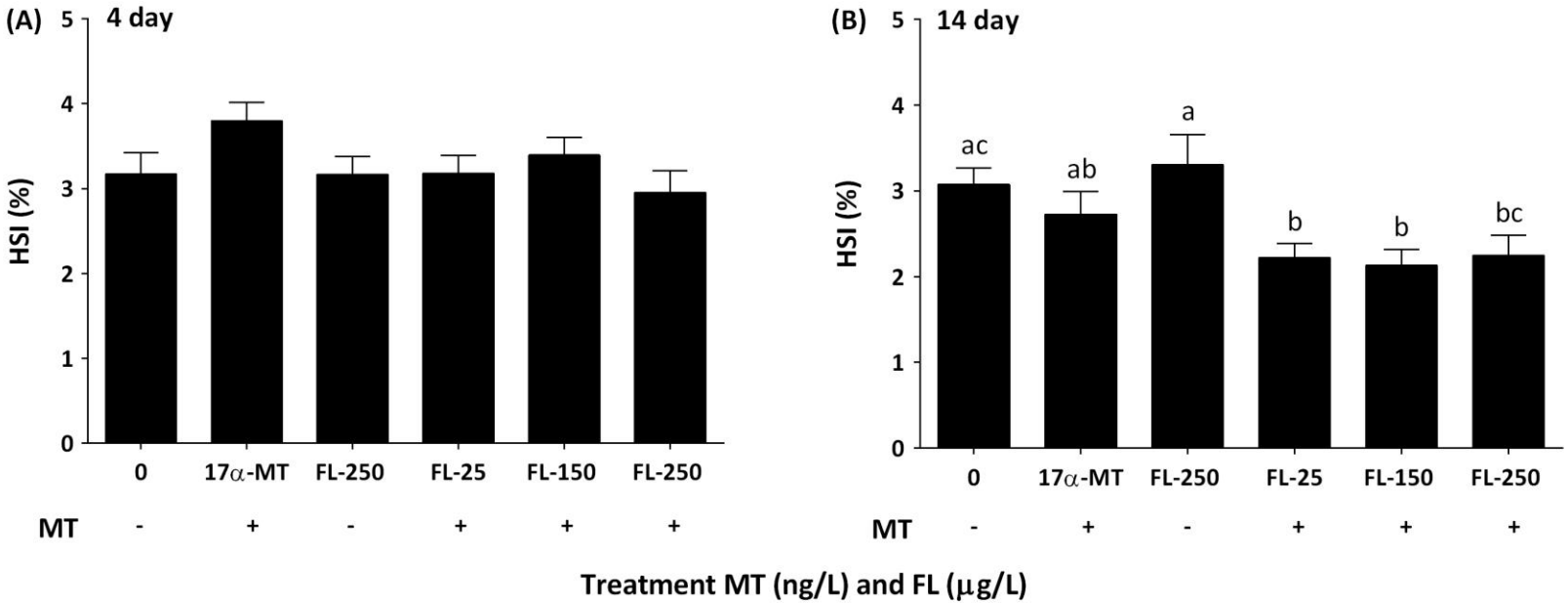

Fig. 3.2 Change in hepatosomatic index (HSI; mean ± SE) in male brook stickleback (*Culaea inconstans*) after (A) 4 days and (B) 14 days of exposure to an acetone-carrier control (0 ng/L), 17a-methyltestosterone (MT: 500 ng/L), flutamide (FL: 250 μg/L) or a combination of MT at 500 ng/L and FL at either 25, 150 and 250 μg/L. Morphometric data was analyzed using a two-way ANOVA with tank nested in treatment and re-run as a one-way ANOVA if the tank effect was not significant ($p \geq 0.250$). Post-hoc analysis was run as a Tukey test. Treatments shown with different letters are significantly different from one another ($p < 0.05$).

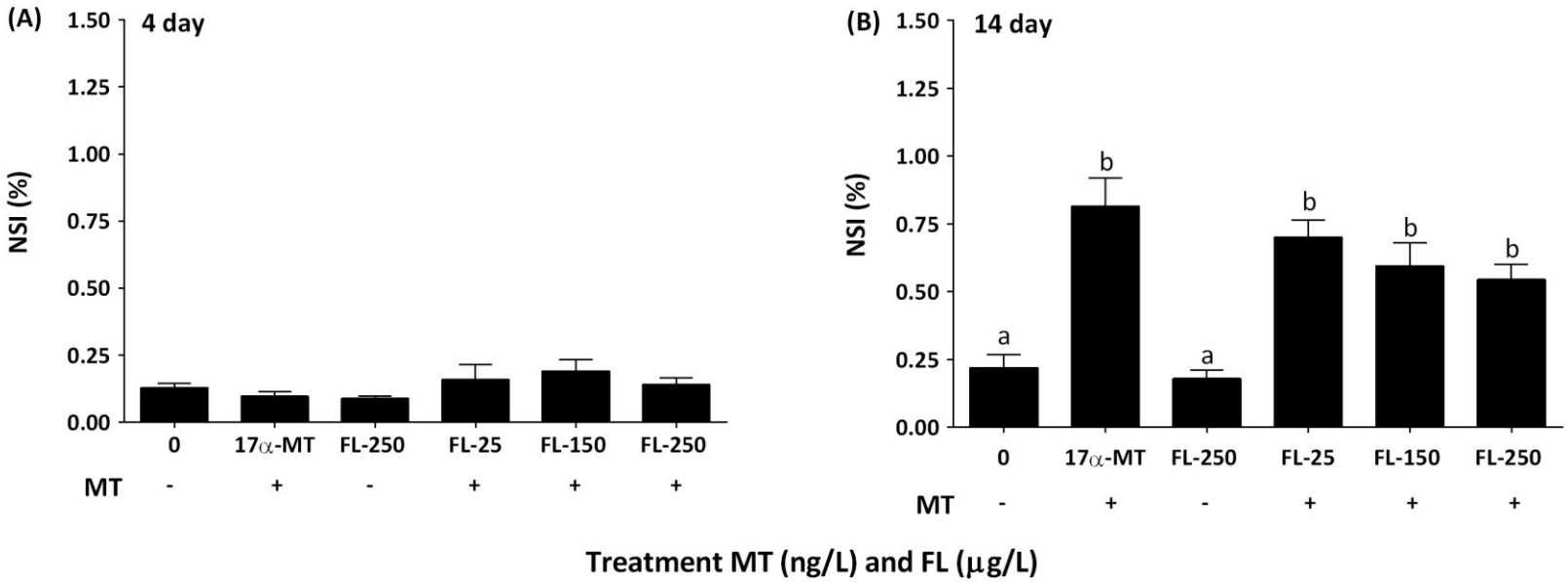

Fig. 3.3 Change in nephrosomatic index (NSI; mean ± SE) in female brook stickleback (*Culaea inconstans*) after (A) 4 days and (B) 14 days of exposure to an acetone-carrier control (0 ng/L), 17a-methyltestosterone (MT: 500 ng/L), flutamide (FL: 250 μg/L) or a combination of MT at 500 ng/L and FL at either 25, 150 and 250 μg/L. Morphometric data was analyzed using a two-way ANOVA with tank nested in treatment and re-run as a one-way ANOVA if the tank effect was not significant ($p \geq 0.250$). Post-hoc analysis was run as a Tukey test. Treatments shown with different letters are significantly different from one another ($p < 0.05$).

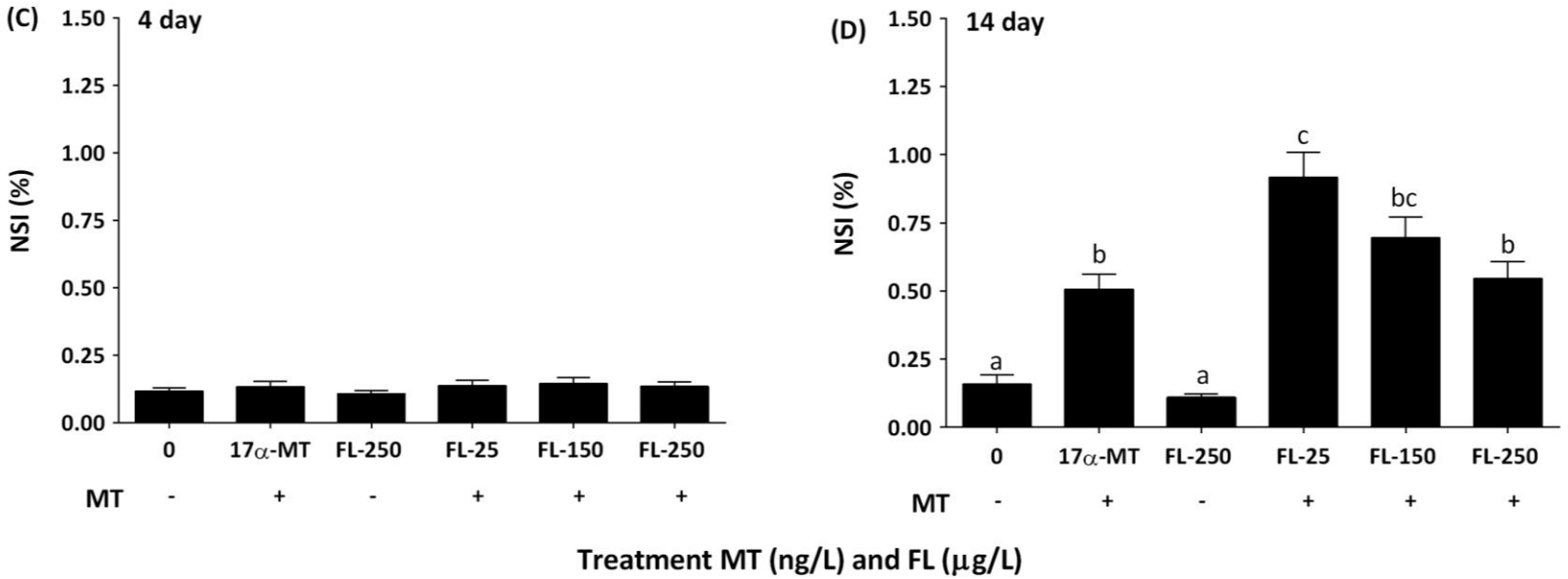

Fig. 3.4 Change in nephrosomatic index (NSI; mean ± SE) in male brook stickleback (*Culaea inconstans*) after (A) 4 days and (B) 14 days of exposure to an acetone-carrier control (0 ng/L), 17a-methyltestosterone (MT: 500 ng/L), flutamide (FL: 250 µg/L) or a combination of MT at 500 ng/L and FL at either 25, 150 and 250 µg/L. Morphometric data was analyzed using a two-way ANOVA with tank nested in treatment and re-run as a one-way ANOVA if the tank effect was not significant ($p \geq 0.250$). Post-hoc analysis was run as a Tukey test. Treatments shown with different letters are significantly different from one another ($p < 0.05$).

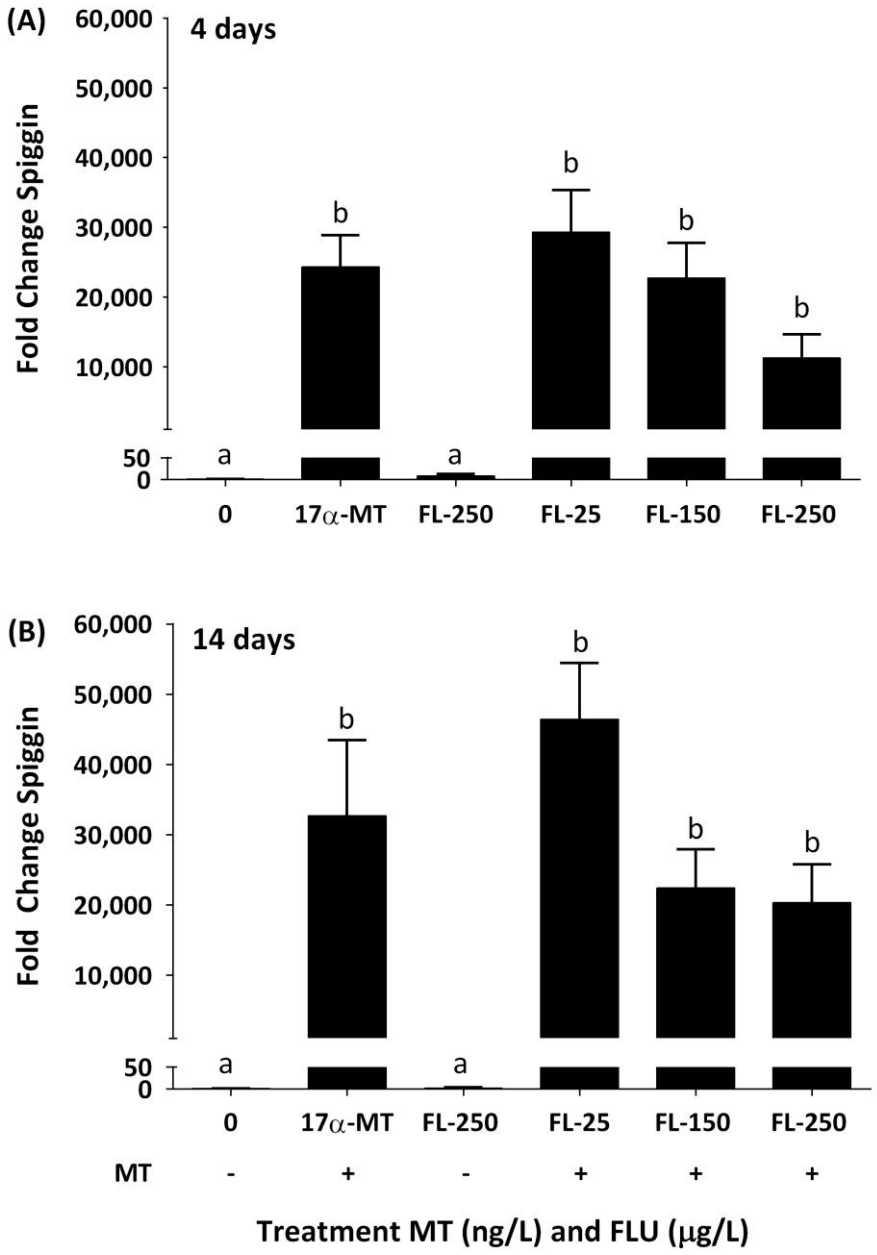

Fig. 3.5 Fold-change of spiggin transcript levels (mean ± SE) relative to acetone-carrier control (0 ng/L) after (A) 4 days and (B) 14 days of co-exposure to 500 ng/L of 17a-methyltestosterone (MT) and flutamide (FL: 25, 150 and 250 μg/L) in the kidney of female brook stickleback (*Culaea inconstans*). MT and FL controls included (17α-MT: 500 ng/L; FL-250: 250 μg/L). Spiggin was measured in duplicate in experimental groups of 4-8 fish. Data was analyzed with a one-way ANOVA and a post-hoc Tukeys test. Treatments shown with different letters are significantly different from one another ($p < 0.05$).

3.3.3 Kidney epithelium cell height

KEH was considerably higher in female sticklebacks exposed to the MT-control (46.7 µm) relative to FL-control (14.4 µm) and solvent-control (15.3 µm) (Fig. 3.6; Table 3.2). KEH slightly decreased in co-treated females at 25 and 250 µg/L FL relative to the MT-control. However, only one sample was available at 25 µg/L FL. KEH was approximately four-times higher in co-treated males relative to solvent- or FL-only controls. Additionally, KEH was slightly lower in males co-treated at 250 µg/L FL relative to 25 µg/L FL (Table 3.2). No samples for male KEH were available in the MT-only control. A statistical analysis to compare among treatment differences in KEH was not be conducted due to limited sample size in some of the treatments (≤ 2 samples/treatment; Table 3.2).

Table 3.2 Mean ± SE (n) of kidney epithelium cell height in female and male brook stickleback (*Culaea inconstans*) exposed to 17α-methyltestosterone and flutamide for 14 days. Treatments are solvent-control (control), 500 ng/L MT positive control (MT-CTRL), 250 µg/L positive control (FL-CTRL) and co-treatments to 500 ng/L MT and 25 µg/L FL (FL-25), 150 µg/L FL (FL-150) or 250 µg/L FL (FL-250).

Sex	Control	MT-CTRL	FL-CTRL	FL-25	FL-150	FL-250
Female	15.3 ± 0.7 (2)	46.7 ± 1.5 (4)	14.4 ± 0.2 (2)	39.1 (1)	46.4 ± 1.8 (3)	41.5 ± 0.9 (2)
Male	16.4 ± 0.3 (2)	-	15.8 (1)	46.3 ± 1.2 (2)	45.2 ± 1.5 (2)	41.8 (1)

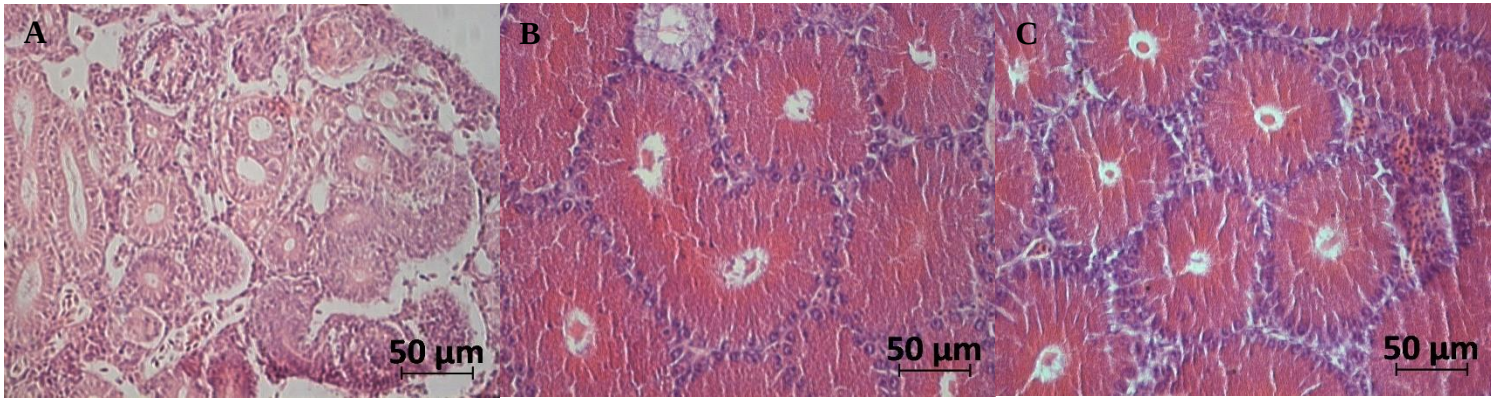

Fig. 3.6 Sections of kidney from female brook stickleback (*Culaea inconstans*) exposed to (A) solvent control (B) 500 ng/L 17α-methyltestosterone (MT) (C) 500 ng/L MT and 250 µg/L flutamide (FL) for 14 days. Sections were cut at 5 µm thickness and stained with hematoxylin-eosin. The scale indicated in the bottom right corner is 50 µm. Kidney epithelium cell height increased from (A) solvent control; KEH = 15.3 µm to (B) MT only; KEH = 46.7 µm and slightly decreased with exposure to (C) MT and FL; KEH = 41.5 µm.

3.4 Discussion

This study is the first to characterize anti-androgenic responses in androgenized brook stickleback. Exposure to FL successfully attenuated the androgen-induced increase in NSI observed in male brook stickleback. A similar reduction in spiggin transcript levels and NSI in androgenized females was observed in FL co-exposed fish although the response was not significant. Nevertheless, this study demonstrated that anti-androgenic responses are measurable with an apical endpoint in the brook stickleback after 14 days of exposure.

This study is the first to document a reduction in NSI in androgenized brook stickleback exposed to FL. NSI was significantly increased with exposure to 500 ng/L MT in both females and males relative to control. An increase in NSI is a reflection of kidney hypertrophy attributed to structural changes in the kidney as it is transformed into a secretory organ (Sokołowska and Kulczykowska, 2006). In a previous study with brook stickleback, 100 ng/L MT did not significantly increase NSI after 21 days of exposure (Muldoon and Hogan, 2015). Therefore, the LOEC for NSI in male brook stickleback is likely > 100 ng/L but may be less than 500 ng/L. In males, NSI in the co-treatments decreased in a concentration-dependent manner with significantly lower NSI at 250 µg/L FL relative to 25 µg/L FL. A reduction in NSI also occurred in androgenized females exposed to increasing concentrations of FL although this was not significant among co-treatments. A reduction in NSI among co-treated males with increased concentrations of FL suggests that FL antagonizes the action of MT. Therefore, changes in NSI may be mediated through the androgen receptor because MT is a potent androgen receptor agonist and flutamide is a potent androgen receptor antagonist (Katsiadaki et al., 2006). A relatively high NSI in the 25 µg/L FL co-treated males compared to the MT-control may reflect inter-individual variability in response to androgen receptor agonists in males.

Although females were androgenized by exposure to MT, their response to FL was variable as indicated by insignificant reductions in NSI among co-treatments. Sex-specific differences in biotransformation may explain the response in female NSI relative to male NSI. FL is an anti-androgenic chemical but its metabolite, hydroxyflutamide (HF), is responsible for the high anti-androgenic potency of FL. HF binds to and blocks the fathead minnow AR with a higher affinity than FL (Ankley et al., 2004). Therefore, FL potency may be affected by the rate at which it is biotransformed to HF. In females, endogenous estradiol (E2) can interfere with biotransformation. For example, in the liver microsomes of humans, E2 reduces the rate of biotransformation of FL to HF through its action on CYP1A2 (Shet et al., 1997). Differences in ethoxyresorufin-O-deethylase (EROD; biomarker for CYP induction) activity between female and male teleost fish has also been attributed to the suppression of CYP1A protein levels by E2 in reproductively mature females whereas EROD activity does not differ between males (regardless of reproductive state) and non-reproductively active females (Whyte et al., 2000). Therefore, we speculate that differences in the reproductive status of individual female fish in this study could have increased inter-individual variability in their response to FL exposure. Inter-individual variation in circulating E2 levels in females could affect biotransformation of FL to HF via the action of E2 on CYP enzymes whereas the biotransformation of FL to HF would be unaffected in males regardless of reproductive status because circulating E2 levels in males are low. However, using female stickleback as the test species rather than males is still preferred because circulating androgen levels in males at specific times of the year can interfere with spiggin response during an anti-androgen exposure (Katsiadaki et al., 2006). Future studies should use fish in a reproductively-regressed state because CYP activity (as indicated by EROD) in non-reproductively active females does not differ from males (Whyte et al., 2000). This could

be achieved by adjusting the photoperiod from 16:8 to 12:12 (light:dark) as is used in the AFSS (OECD, 2011).

Although spiggin production in threespine stickleback is effectively reduced with exposure to anti-androgenic chemicals (Hogan et al., 2012; Katsiadaki et al., 2006), such a significant response was not observed in brook stickleback tested in the present study. Exposure to increasing FL concentrations reduced spiggin transcript levels in androgenized female brook stickleback but the high inter-individual variability limited the ability to discern significant differences among treatments. FL exposure has been previously reported to significantly inhibit spiggin protein production in androgenized female threespine stickleback at 25 µg/L FL (Katsiadaki et al., 2006) and 50 µg/L FL (Jolly et al., 2009). However, high inter-individual variability meant that Jolly et al. (2009) were also unable to detect a significant reduction in spiggin protein production in androgenized female threespine stickleback at 25 µg/L FL, despite a 50 percent decrease in production relative to the positive control.

Other factors such as chemical potency and exposure duration may account for the lack of antagonism by FL as measured by spiggin mRNA levels in androgenized brook stickleback. Brook stickleback were exposed to 500 ng/L MT to androgenize females yet this concentration may be too potent to be antagonized by FL, especially considering only 100 ng/L MT was required to elicit an androgenic response (Chapter 2). In a previous study with threespine stickleback, FL did not inhibit spiggin protein production in females androgenized with 5 µg/L MT (Katsiadaki et al., 2006). It was concluded that FL antagonism was not possible with 5 µg/L MT due to the potency of MT (Katsiadaki et al., 2006). Moreover, some androgenic compounds are more readily antagonized by FL than others and MT may not be the appropriate androgen to masculinize female brook stickleback. For example, Katsiadaki et al. (2006) found that

dihydrotestosterone (DHT) was antagonized more by FL than MT in threespine stickleback; a significant reduction in spiggin protein, in threespine stickleback androgenized with DHT, occurred with exposure to 10 µg/L FL. However, DHT is less stable in water than MT and requires flow-through conditions to maintain stable concentrations (Katsiadaki et al., 2006). Furthermore, differences in exposure duration may have affected the ability of FL to antagonize MT-induced spiggin production in threespine relative to brook stickleback; the length of exposure in this study was 14 days whereas comparable studies with threespine stickleback were conducted for 21 days (Jolly et al., 2009; Katsiadaki et al., 2006). An exposure period longer than 14 days may be required to biotransform FL to HF in concentrations adequate enough to antagonize MT.

KEH was previously found to respond to exposure to both androgenic and anti-androgenic chemicals in threespine stickleback (Katsiadaki et al., 2006) and our results indicate that the KEH in brook stickleback may be a potentially useful biomarker of exposure to anti-androgens but additional samples are needed to adequately test for an anti-androgenic response. KEH increased substantially (almost four-times that of the solvent control) as a result of exposure to MT. Increased KEH is due to spiggin production in the secondary proximal tubules (Jakobsson et al., 1999; Katsiadaki et al., 2002b). KEH in female brook stickleback exposed to 500 ng/L MT (46 µm) was higher than KEH in female threespine stickleback (28 µm) exposed under similar conditions (Katsiadaki et al. 2006). However, KEH in wild male threespine stickleback has been recorded to be as high as approximately 42 µm (Sokolowska and Kulczykowska, 2006). Therefore, a KEH of 46 µm in female brook stickleback is physiologically realistic. The disparity in KEH between MT-exposed brook and threespine stickleback may hint at a difference in the species sensitivity in responding to MT.

There was no effect of FL- or MT-only exposure on HSI in brook stickleback but HSI was significantly reduced when the compounds were administered in combination. In fish, the liver plays a critical role in energy storage and metabolism so changes in HSI can indicate impacts on liver function (reviewed in Schmitt and Dethloff, 2000). Reduced HSI may be due to additive or synergistic effects of the chemical mixture versus single-compound exposure. Reduced HSI has been observed in fish exposed to complex mixtures such as pulp and paper mill effluent (Adams et al., 1992; McMaster et al., 1991). Adams et al. (1992) suggested that decreased HSI in fish could be caused by energy depletion due to increased energy metabolism. For example, depleted energy-stores (glycogen) in rainbow trout were measured alongside reduced HSI and attributed to increased metabolism in response to chronic cortisol exposure (Barton et al., 1987). Therefore reduced HSI in co-treated brook stickleback may be attributed to reduced energy stores from increased metabolic demands associated with exposure to multiple chemicals. However, this mechanism cannot be confirmed without measuring metabolic endpoints.

Anti-androgen bioassays have been developed in other small model fish species, albeit with lower biological relevance or sensitivity compared to the stickleback. Recently, Sébillot et al. (2014) designed an anti-androgen assay in transgenic juvenile medaka in which the spiggin gene was fused to a green fluorescent protein and changes in spiggin expression were monitored by measuring fluorescence. Spiggin production was induced with exposure to 3 µg/L MT and significantly reduced in medaka co-exposed to > 138 µg/L FL (Sébillot et al., 2014). However, the transgenic medaka assay lacks biological relevance and for that reason is meant to serve as a screening tool for anti-androgenic chemicals rather than to predict their effect (Sébillot et al., 2014). In the fathead minnow, a reduction in nuptial tubercles in androgenized females is used to

detect anti-androgenic effects (Ankley et al., 2004; Jensen et al., 2004; Martinović et al., 2008). Unlike this study, anti-androgen assays with fathead minnow use 17β-trenbolone because the MT concentration required to masculinize females causes estrogenic effects due to the aromatization of MT to methylestradiol (Hornung et al., 2004). In female fathead minnow androgenized with 500 ng/L 17β-trenbolone, a reduction in nuptial tubercles followed co-exposure to 400 µg/L of FL (Ankley et al., 2004). Nuptial tubercle reduction in male fathead minnow has also been measured following exposure to FL. Nuptial tubercles were unaffected at 651 µg/L FL (Jensen et al., 2004) or 350 µg/L FL (Filby et al., 2007) but were reduced following exposure to 938 µg/L FL (Panter et al., 2004). Therefore, a reduction in nuptial tubercles in androgenized female or non-androgenized male fathead minnows requires ≥ 400 µg/L FL or > 651 µg/L FL, respectively. In contrast, 250 µg/L FL resulted in a significant reduction in NSI in male brook stickleback. In addition, after 14 days exposure to 150 µg/L FL a slight (but insignificant) downward trend in spiggin transcript levels was evident in androgenized female brook stickleback. KEH also appeared to be lower in the 250 µg/L FL co-treated females. However, further testing with additional anti-androgenic compounds in brook stickleback is needed to thoroughly characterize their response.

Brook stickleback also hold potential as a bioindicator for anti-androgenic activity through the exploitation of endpoints other than spiggin, KEH and NSI. Several studies have cultured this species in the lab (McKenzie, 1969; McLennan, 1993; Reisman and Cade, 1967) and therefore, apical endpoints not measured here, such as reproductive behaviour, could be measured in brook stickleback to determine if a relationship exists between impaired spiggin production and aberrant behaviour. Sebire et al. (2008) developed an assay to measure behavioural impairments in photoperiodically-stimulated male threespine stickleback and found

that significantly fewer nests were present and courtship behaviour was impaired with exposure to 100 and 500 µg/L FL while 500 µg/L FL also impaired nest building behaviour (e.g. digging). Impaired behaviour was accompanied by an inhibition in photoperiodically-stimulated spiggin production in males at ≥ 500 µg/L FL (Sebire et al., 2008). Therefore, impaired spiggin production may be associated with behavioural effects that could ultimately influence reproduction. Fecundity is another biologically-relevant and responsive endpoint measured in anti-androgen assays with fathead minnow. Exposure to 650 and 500 µg/L FL in female fathead minnow resulted in reduced total egg production (Battelle, 2003) and reduced number of eggs per spawn and number of spawns per female (Jensen et al., 2004), respectively. Whether exposure to an anti-androgen would impair fecundity in breeding stickleback is unknown but this endpoint could be incorporated into future assays. These proposed reproductive assays could be coupled with existing *in vitro* assays using brook stickleback that could provide a rapid cost-effective tool for initial screening of anti-androgenic chemicals. An *in vitro* kidney primary culture cell assay in threespine stickleback is the most sensitive method (to date) in stickleback used to detect anti-androgens; spiggin was reduced in DHT-treated cells at 0.28 ng/L FL (Jolly et al., 2009). Moreover, this *in vitro* assay produced similar results as a concurrently conducted *in vivo* assay but with 550 less fish and in three days versus 21 days (Jolly et al., 2009).

3.5 Conclusion

In conclusion, the present study is the first to measure an anti-androgenic response in brook stickleback and demonstrates the applicability of this species as a bioindicator for anti-androgenic chemicals using the biomarkers measured in this study. We demonstrated an anti-androgenic response through reduced NSI in androgenized brook stickleback. While we were unable to detect a difference among co-treatments for spiggin inhibition in androgenized females

due to inter-individual variability, we hypothesize that exposing female stickleback in a reproductively quiescent state would reduce this variability. In addition, future studies should focus on testing a combination of different androgen-priming compounds for co-exposure with FL to determine the most suitable androgen that can be adequately antagonized by FL in a concentration-dependent manner.

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CHAPTER 4

4. GENERAL DISCUSSION

4.1 Introduction

Considerable research has focussed on identifying compounds that can disrupt reproduction, development and behaviour in aquatic organisms (Ankley and Johnson, 2004; Arukwe, 2001; Mills and Chichester, 2005). Documented cases of endocrine disruption have been noted in wild and laboratory fish exposed to EDCs, including overt effects such as feminization and demasculinization as well as more subtle changes such as the uncharacteristic production of sex-specific proteins (Arukwe, 2001). The vast majority of studies have thus far focussed on identifying and characterizing EDCs that target estrogen-dependent pathways (Sumpter and Johnson, 2008). However, EDCs with (anti-)androgenic properties are now believed to be ubiquitous within the aquatic environment (e.g. from WWTPE, PPME and runoff from livestock operations) and may be important contributors to reproductive dysfunction in aquatic animals (Bartelt-Hunt et al., 2012; Ellis et al., 2003; Howell et al., 1980; Jobling et al., 2009; Schiffer et al., 2001). Despite this attention, the biological significance of (anti)-androgenic contaminants is not fully understood, in part due to a lack of (anti-)androgen-specific biomarkers in fish. Consequently, little is known about the identity of (anti-)androgenic EDCs or the extent of their effects (if any) on aquatic wildlife, highlighting a need for further investigation and assessment.

Small model fish are exploited in the testing of EDCs because of their small size, short life, rapid reproductive cycle and the availability of a suite of biomarkers that can be integrated to evaluate the toxicity of EDCs (Scholz and Mayer, 2008). Fathead minnow, Japanese medaka,

zebrafish and threespine stickleback are small fish models commonly used to evaluate EDCs. All four species have estrogen-responsive biomarkers (e.g. vitellogenin), which have facilitated the identification and prioritization of chemicals with estrogenic-activity that may be contributing to endocrine disruption in aquatic wildlife. Alternatively, only threespine stickleback have a sensitive and quantifiable (anti-)androgenic biomarker (spiggin) that is robust in detecting chemicals with (anti-)androgenic activity (Katsiadaki and Sebire, 2011). The application of small model fish in EDC biomonitoring in the field limits the use of species to regions in which they are endemic. The threespine stickleback's geographical distribution is limited to coastal and estuarine habitats in North America, which prevents their use for EDC biomonitoring in freshwater environments in the continent. Brook stickleback are a small fish that inhabit freshwater environments throughout North America and, like threespine stickleback, males also possess the (anti-)androgen responsive gene, spiggin. Therefore, the objective of this thesis was to develop brook stickleback as a bioindicator species for EDCs by evaluating their response and sensitivity to estrogenic and (anti-)androgenic chemical exposure.

Spiggin and vitellogenin have never previously been measured in brook stickleback and so, a qPCR assay was developed with the purpose of measuring and characterizing the sex-specific expression of these endpoints in mature wild-caught brook stickleback. As was predicted, spiggin and vitellogenin were differentially expressed in male versus female brook stickleback (Chapter 2), although the magnitude of difference between the sexes was smaller compared to studies with threespine stickleback. The second study (Chapter 2) was designed to determine the response and sensitivity of brook stickleback to a model androgen (MT) and estrogen (EE2) using biomarkers spanning multiple biological levels. The results of this study illustrated that biomarkers in brook stickleback were highly responsive to EE2 and MT exposure

and followed a similar pattern of response to other small fish models. As was predicted, MT exposure induced spiggin expression and increased KEH in female brook stickleback whereas EE2 exposure induced vitellogenin expression in males and increased HSI in males and females. NSI was unaffected by exposure to MT, and may not be a robust biomarker for androgenic chemicals in brook stickleback, whereas GSI and kidney histopathology were affected by EE2 and could be useful in evaluating the toxicity of estrogenic chemicals in future studies. Therefore, this study illustrated that molecular, histological and morphological biomarkers are available for the evaluation of androgenic and estrogenic compounds using brook stickleback. The third study (Chapter 3) sought to determine the response and sensitivity of brook stickleback exposed to a model anti-androgenic chemical using the biomarkers developed and tested in the previous study. An anti-androgenic response was demonstrated through reduced NSI in androgenized males co-exposed to FL whereas a slight suppression of spiggin mRNA levels and a trend towards decreased NSI in androgenized females co-exposed to FL suggested a similar anti-androgenic response. High inter-individual variability within treatments prevented the statistical evaluation of this response although it closely resembled the pattern of effects observed in previous studies with the threespine stickleback (Katsiadaki et al., 2006; Katsiadaki and Sebire, 2011).

4.2 Comparing interspecies sensitivities among small fish models

Cross-species comparisons are used to evaluate the relative sensitivity of biomarkers to chemical exposure as a method to validate small fish models as bioindicators for EDCs. Several studies have compared estrogenic and (anti-)androgenic responses among threespine stickleback, Japanese medaka, zebrafish and/or fathead minnow to identify sensitive and specific biomarkers for the various classes of EDCs (Katsiadaki and Sebire, 2011; Lange et al., 2012; OECD, 2006;

Seki et al., 2006). The results in this thesis were the first to report (anti-)androgenic and estrogenic responses in brook stickleback exposed to model EDCs and thus, cross-species comparisons between brook stickleback and small model fish are not available in the literature. Therefore, one of the objectives of this thesis was to determine the sensitivity of brook stickleback to estrogenic and (anti-)androgenic model compounds by comparing its biomarker responses to those in other small fish models exposed to model EDCs. Although zebrafish are used as model fish for estrogenic compounds they do not possess an androgenic-specific biomarker so they are excluded from discussion.

4.2.1 Comparison to threespine stickleback

The studies conducted in this thesis demonstrated that exposure to an estrogenic or androgenic chemical produced comparable responses in brook and threespine stickleback, notwithstanding a slightly lower sensitivity in brook stickleback. Biomarker responses in brook stickleback exposed to EE2 and MT followed a similar pattern as threespine, when compared to studies in the literature (Chapter 2). In threespine and brook stickleback, spiggin in females and vitellogenin in males increased in response to MT and EE2 exposure, respectively (Table 4.1; Table 4.2). Changes at higher levels of biological organization (HSI and KEH) were also measurable with exposure to androgenic and estrogenic compounds in both stickleback species. However, a higher concentration of MT and EE2 was required to elicit a molecular response in brook relative to threespine stickleback (Table 4.1; Table 4.2). Brook stickleback were also not as responsive to anti-androgenic chemical exposure compared to the threespine stickleback. Androgen-induced spiggin in female brook stickleback was not significantly reduced with co-exposure to FL even at the highest tested FL concentration, whereas a similar study with androgenized female threespine stickleback reported reduced spiggin production at a relatively

low FL concentration (Katsiadaki et al., 2006; Table 4.3). NSI in androgenized male brook stickleback was significantly reduced with exposure to FL but, because this endpoint has not been reported in anti-androgenic exposures with threespine stickleback, no comparison of NSI between these species can be made (Table 4.3).

Several factors could explain the apparent lower sensitivity of brook stickleback, in responding to the test chemicals, relative to threespine stickleback. The greater sensitivity of threespine relative to brook stickleback may be due to differences at the molecular level that influence ligand-receptor binding affinity, receptor expression, chemical affinity to steroid binding globulins, as well as chemical uptake and clearance (Lange et al., 2012; Wells and Van Der Kraak, 2000). Differences in exposure conditions, including time of year, photoperiod, water temperature and water flow rates can affect chemical uptake, metabolism and clearance (Katsiadaki et al., 2006) and may also explain varied sensitivity between the stickleback species. Side-by-side exposures with brook and threespine stickleback would allow for a direct comparison of biomarker responses between the species while controlling for exposure conditions. Lastly, the sensitivity of brook stickleback to androgenic and estrogenic chemicals may more closely resemble threespine stickleback, which could be tested in future studies through the establishment of a more precise dose-response for spiggin and vitellogenin.

4.2.2 Comparison to fathead minnow and medaka

As discussed throughout this thesis, both fathead minnow and medaka have morphological responses that can be measured to evaluate (anti-)androgenic chemicals, although they lack specificity and sensitivity (Katsiadaki and Sebire, 2011). The biomarker responses of brook stickleback to (anti-)androgenic EDCs were ranked as more sensitive compared to fathead minnow which suggests that brook stickleback may be better suited for detecting low levels of

(anti-)androgenic activity in the freshwaters of North America. The threshold for nuptial tubercle formation in female fathead minnow exposed to MT was approximately 10-times higher than spiggin induction in female brook stickleback (Table 4.1). Similarly, the threshold for nuptial tubercle suppression in androgenized female fathead minnow exposed to FL was twice as high as NSI suppression in androgenized male brook stickleback (Table 4.3). Although changes in papillary processes in medaka are more sensitive to androgen exposure (specifically MT) when compared to spiggin mRNA induction in brook stickleback (Table 4.1), a refined dose-response for induction of spiggin mRNA could decrease its response threshold, thereby increasing the reported sensitivity of brook stickleback to androgen exposure. It is important to note that an androgenized female medaka assay for testing anti-androgenic chemicals has not yet been conducted. Nevertheless, based on the evidence currently available, papillary processes in medaka are not responsive to anti-androgens (Nakamura et al., 2014; OECD, 2006). Overall, with further refinement of the exposure assay, NSI and spiggin in brook stickleback hold excellent potential as biomarkers for (anti-)androgenic chemicals.

The chemical potency of (anti-)androgens may be better predicted by quantifying spiggin mRNA expression compared to secondary sexual characteristics because of the narrow dynamic range of the latter biomarker. The results of this thesis demonstrated a wide dynamic range for spiggin induction in brook stickleback. In the MT study, spiggin induction in brook stickleback spanned four orders of magnitude whereas (in comparable studies) the induction of secondary sexual characteristics in female fathead minnow and medaka spanned less than two orders of magnitude - from 0 to 85 papillary process and 0 to 11 nuptial tubercles in medaka and fathead minnow, respectively(Kang et al., 2008; Pawlowski et al., 2004a). The wide dynamic range and sensitivity of spiggin may also make it a more suitable biomarker for identifying androgenic

chemicals with non-monotonic dose-response relationships, which are common for estrogenic EDCs (Zoeller and Vandenberg, 2015).

Measurement of vitellogenin in fish is extensively used as a biomarker for exposure to compounds with estrogenic activity. One study compared the response of vitellogenin among model fish species exposed to EE2 and ranked species in order of sensitivity: zebrafish > fathead minnow > medaka > threespine stickleback (Lange et al., 2012). This rank order of sensitivity is also indicated in Table 4.2 where it is clear that vitellogenin induction is a more sensitive indicator of estrogenic exposure in fathead minnow and medaka (compared to brook or threespine stickleback) and thus, these species may be more adequately suited to detecting environmentally-relevant concentrations of estrogenic chemicals which typically range from 1-50 ng/L (reviewed in Mills and Chichester, 2005). However, the advantage of stickleback over fathead minnow and medaka remains the potential to simultaneously assess (anti-)androgenic and estrogenic chemical exposure using a single species.

Table 4.1 Differences in sensitivity among small-bodied model fish species and female brook stickleback (*Culaea inconstans)* exposed to 17α-methyltestosterone.

Endpoint	*Culaea inconstans*		*Gasterosteus aculeatus*		*Pimephales promelas*		*Oryzias latipes*	
	NOEC	LOEC	NOEC	LOEC	NOEC	LOEC	NOEC	LOEC
Spiggin	10	100	1 [a]	10 [a]	N/A	N/A	N/A	N/A
SSC	N/A	N/A	N/A	N/A	100 NT [d]	1000 NT [d]	0 PP [e]	22.5 PP [e]
NSI	100	> 100	< 100 [b]	100 [b]	N/A	N/A	N/A	N/A
KEH	10 *	100 *	10 [c]	100 [c]	N/A	N/A	N/A	N/A

LOEC and NOEC values provided in ng/L. Data from [a]Hogan et al., 2008; [b]Sanchez et al., 2008b; [c]Katsiadaki et al., 2002b; [d]Pawlowski et al., 2004; [e]Kang et al., 2008. Secondary sexual characteristics (SSC); nephrosomatic index (NSI); kidney epithelium cell height (KEH); nuptial tubercles (NT); papillary processes (PP); not measured (N/A). *not statistically analyzed due to insufficient sample size.

Table 4.2 Differences in sensitivity among small-bodied model fish species and female brook stickleback (*Culaea inconstans)* exposed to 17α-ethinylestradiol.

Endpoint	*Culaea inconstans*		*Gasterosteus aculeatus*		*Pimephales promelas*		*Oryzias latipes*	
	NOEC	LOEC	NOEC	LOEC	NOEC	LOEC	NOEC	LOEC
Vitellogenin	10*	100*	6 [a*]	18 [a*]	3 [c]	10 [c]	0.2 [d]	2 [d]
HSI	10	100	51 [b]	170 [b]	N/A	N/A	0.2 [d]	2 [d]

LOEC and NOEC values provided in ng/L. Data from [a]Katsiadaki et al., 2010; [b]Andersson et al., 2007; [c]Pawlowski et al., 2004; [d]Ma et al., 2007. Hepatosomatic index (HSI); not measured (N/A). *measured as mRNA rather than protein.

Table 4.3 Differences in sensitivity among small-bodied model fish species and female brook stickleback (*Culaea inconstans)* exposed to flutamide.

	Culaea inconstans		*Gasterosteus aculeatus*		*Pimephales promelas*		*Oryzias latipes*	
Endpoint	NOEC	LOEC	NOEC	LOEC	NOEC	LOEC	NOEC	LOEC
Spiggin	250	> 250	< 25	25 [a]	N/A	N/A	N/A	N/A
SSC	N/A	N/A	N/A	N/A	< 400 NT	400 NT [b]	N/A	> 1000 PP [c]
NSI	150	250	N/A	N/A	N/A	N/A	N/A	N/A
KEH	150 *	250 *	< 500 †	500 [a] †	N/A	N/A	N/A	N/A

LOEC and NOEC values provided in µg/L. Data from [a]Katsiadaki et al., 2006; [b]Ankley et al., 2004; [c]OECD, 2006. * not statistically analyzed due to insufficient sample size. *Culaea inconstans* and *Gasterosteus aculeatus* co-exposed to flutamide and 17α-methyltestosterone at 500 ng/L except † exposed at 10 µg/L. *Pimephales promelas* co-exposed to flutamide and 500 ng/L 17β-trenbolone. *Oryzias latipes* exposed only to flutamide. Secondary sexual characteristics (SSC); nephrosomatic index (NSI); kidney epithelium cell height (KEH).

4.3 Future research directions

The research presented in this thesis provides the first documented response of brook stickleback to EDC exposure and as such, can be used as a foundation upon which to design future studies. Although the results of this thesis demonstrate that brook stickleback are responsive to (anti-)androgenic and estrogenic chemicals, additional research is necessary to further develop this species as a bioindicator for EDCs in the aquatic environment.

The sensitivity of biomarker responses in brook stickleback could be improved with further refinement of exposure conditions, which would also ease the comparability of this species to other small fish models. In the MT and EE2 exposures (Chapter 2), it was demonstrated that although spiggin and vitellogenin were responsive to hormone exposure, they had a higher response threshold when compared to the same biomarkers in threespine. Future studies should focus on refining the response of molecular biomarkers in brook stickleback by incorporating exposure concentrations between 10 to 100 ng/L for MT and EE2. In the case of KEH, data were based on a limited sample size due to the prioritization of kidney samples for molecular analysis. Future research could allocate additional samples for histology to confirm the response of this biomarker to androgenic chemicals. High inter-individual variability likely led to a lack of effect of FL on NSI or spiggin expression in androgenized female brook stickleback, even though effect sizes were large (20-60% reduction in spiggin mRNA in androgenized females; Chapter 3). Inter-individual variability could be reduced in future anti-androgenic studies by substituting the AR agonist (with a chemical other than MT) or reducing its concentration (< 500 ng/L) to allow FL to more effectively antagonize MT. Because estradiol (E2) levels can interfere with chemical metabolism (Shet et al., 1997; Whyte et al., 2000) future studies with FL should also reduce the exposure photoperiod to 12:12 (light:dark) so females are

in a reproductively quiescent state (low E2) and extend the exposure time to 21 days to allow for adequate conversion of FL to its more active metabolite hydroxyflutamide.

In other small fish models, studies on mechanisms of action (both *in vitro* and *in vivo*) provide insight into differences in interspecies sensitivity and improve the extrapolation of molecular changes in predicting apical effects (relevant for ecological risk assessment). There is no information in the literature on receptor affinity, receptor expression or sex steroid levels in brook stickleback. Therefore, future studies could measure the affinity of test chemicals to AR and ER receptors as well as the expression of AR and ER and levels of E2, testosterone and 11-ketotestosterone in brook stickleback. Receptor expression and sex steroid levels would be valuable in determining the mechanisms underlying endocrine disruption in brook stickleback whereas binding affinity assays could help explain the varied sensitivity to specific EDCs between this and other species. Future studies could also test *in vitro* assays, using kidney explants or cells in brook stickleback, as screening tools to detect (anti-)androgenic activity (Björkblom et al., 2007; Jolly et al., 2009, 2006).

Although diagnostic biomarkers were evaluated in the studies herein, apical endpoints are also valuable in evaluating the toxicity of EDCs and necessary to link molecular biomarkers to ecologically-relevant endpoints that can be incorporated into risk assessment. Future research directions could involve establishing reproductive assays with brook stickleback that include measurements of fecundity and fertility that could then be correlated with spiggin induction or suppression and changes in KEH or NSI. Furthermore, behavioural assays could be designed in future studies to measure the effect of (anti-)androgenic chemicals on male nesting and courting behaviour, which could also be associated to changes in spiggin, and used to evaluate potential effects of EDC exposure in this species.

To utilize brook stickleback in hazard testing, and potentially in biomonitoring, biomarker responses in this species should be evaluated under environmentally-relevant exposure scenarios. The scope and applicability of brook stickleback as a bioindicator species could be demonstrated through the evaluation of biomarker responses to environmentally-relevant EDCs, including progestins (e.g. levonorgestrel; Svensson et al., 2013), livestock-operation pollutants (e.g. 17β-trenbolone; Allen et al., 2008) and pesticides (e.g. linuron and vinclozolin; Hogan et al., 2012; Jolly et al., 2009). Brook stickleback also have potential to be used as biomonitoring species to detect androgenic activity in whole-effluent and in effluent-receiving waters, so future research could test responses in brook stickleback under these exposure scenarios. However, to accurately evaluate the data obtained in field exposures, future research should first determine the seasonal variability of spiggin, vitellogenin, OSIs, KEH and CF in brook stickleback.

One of the challenges to be addressed by future studies will be incorporating a brook stickleback AFSS for biomonitoring for anti-androgenic activity in the field. Although the AFSS can be applied to monitor for endocrine-disrupting activity of whole-effluent in a laboratory, it is not possible to incorporate (as is) in the field. However, field-based artificial streams could be used to incorporate the AFSS into field biomonitoring studies. An artificial stream is created by circulating water from an effluent-receiving environment through mesocosms (in which fish are housed) located inside a controlled and enclosed laboratory next to a stream or river. This method has been calibrated and employed in the testing of endocrine disruption from PPME exposure in eastern Canada and is environmentally-relevant and cost-effective (Dubé et al., 2002). The AFSS also requires that females be androgenized but exposing the fish to a waterborne androgen could be detrimental to the water quality in the receiving environment.

Therefore, fish housed in the mesocosms could be androgenized using surgically implanted silicon tubing which contains an androgenic-chemical as described by Olsson et al. (2005). Using this method, females would be androgenized without compromising the water quality of the receiving environment.

4.4 Conclusion

This thesis successfully demonstrated that brook stickleback possess biomarkers that are responsive to synthetic hormone exposure. This research was the first to document the presence and differential expression of basal spiggin and vitellogenin transcripts in mature female and male brook stickleback. The response of brook stickleback to model compounds mirrored that of threespine stickleback suggesting that they may have similar applicability in the assessment and evaluation of (anti-)androgenic chemicals. Although higher exposure concentrations were required to initiate a biomarker response in brook relative to threespine stickleback, refined exposure conditions could lower the threshold for molecular responses in brook stickleback. More importantly, spiggin and NSI in brook stickleback were among the more sensitive biomarkers for (anti-)androgenic chemicals relative to comparable biomarkers in other established small fish model species. Although spiggin mRNA was not suppressed with co-exposure to FL in androgenized females, this was likely due to high inter-individual variability, which may be reduced with modifications to exposure conditions. Brook stickleback did not display a greater sensitivity to EE2 compared to medaka or fathead minnow and yet, the ability to simultaneously assess estrogenic and (anti-)androgenic chemical exposure in a single fish using quantitative endpoints is an advantage exclusively held by members of the stickleback family. Overall, the confirmation of (anti-)androgen–specific responses in brook stickleback

position this species as a potentially useful bioindicator for testing EDCs as well as for biomonitoring for endocrine-active compounds in North American freshwaters.

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